Identification of Biologically Active Factors in the Ionizing Radiation Regulated Secretome

The Role of Placental Growth Factor

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

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Zürich, 2020

Abstract

Both lung cancer and medulloblastomas are treated with ionizing radiation (IR) in combination with surgery or anticancer agents. Although the survival rates have improved over the past decades, both of these cancer types still demonstrate a poor overall survival and long-term side effects are common, especially for medulloblastoma in children. One of the main hurdles for successful treatment outcome in cancer patients is the acquired resistance to treatment. As a stress mechanism in response to IR, cancer cells secrete several growth factors that can act in an auto- and paracrine manner and, thereby, can support tumor growth. One of these factors is placental growth factor (PlGF) which is mainly involved in tumor angiogenesis.

PlGF expression is increased in cancer patients in response to various treatment forms. Even though its role in pathological angiogenesis has been shown before, not much attention has been directed to PlGF and our knowledge about the role of PlGF for treatment resistance is limited. Clinical trials with neutralizing antibodies targeting PlGF in combination with other anti-cancer agents was discontinued due to lack of improved survival in previously treated patients. The regulation of PlGF in response to ionizing radiation and its role for the treatment response to radiotherapy has not been investigated so far. Thus, further research focusing on PlGF as a potent molecular target as part of a combined treatment modality with ionizing radiation is needed.

In this PhD thesis, we investigated PlGF expression and secretion in several cancer cell lines and provide insight into IR-induced regulation of PlGF. Our additional experiments focused on determining the role of PlGF as a rescue mechanism in response to IR.

Based on results from our bioplex cancer biomarker assay, we investigated PlGF expression and secretion at early and late time points in response to increasing doses of IR in multiple cancer cell lines. Early PlGF expression correlated with early secretion in p53 wildtype cells. Delayed PlGF expression and secretion in p53 mutated cells could depend on other transcription factors, such as hypoxia inducible factors (HIFs) and subsequent downstream signaling pathways. The mechanism behind IR-induced PlGF signal transduction pathway is not fully understood. In order to verify PlGF regulation, we performed mechanistic investigations by genetic and

pharmacologic targeting of p53. Our results demonstrated a clear and specific PlGF regulation by wildtype p53. In parallel, the expression and secretion of PlGF alone, and in combination with irradiation under hypoxia was investigated to show a potential differential regulation of PlGF in p53 mutated cells by HIF-1α. We show increased PlGF secretion under hypoxic conditions in combination with IR. However, this was abolished in cells treated with the HIF-1α inhibitor BAY 87-2243.

The second part of the project investigated the protective role of PlGF in tumor angiogenesis and radiation therapy.

In our *in vitro* experiments, we investigated the paracrine effect of PlGF in a migration assay using PlGF wildtype or knockout cells. We assessed the migratory capacity of human umbilical endothelial vein cells (HUVECs) towards PlGF-wt and PlGF-ko cells in response to IR. Increased migratory capacity of HUVECs towards irradiated PlGF-wt cells versus PlGF-ko cells was observed. We also investigated the role of PlGF on the formation of the tumor vasculature and tumor radiosensitivity *in vivo*. Mice bearing PlGF-ko tumors demonstrated decreased vascular integrity in response to irradiation and increased radiosensitivity. 60% of mice with PlGF-ko tumors regressed completely after single high dose irradiation.

In conclusion, my PhD project provides first insights into genetic background-dependent regulation of PlGF as part of a treatment response to ionizing radiation. The obtained results also demonstrate an important IR-induced paracrine role of PlGF in vessel protection and treatment resistance. Based on our *in vitro* and *in vivo* results, we propose PlGF to be of translational interest in tumors with wildtype p53 background and a promising target for a combined treatment modality in combination with ionizing radiation.

List of Abbreviations

ADC	Antibody-drug conjugates
ALK	Anaplastic lymphoma kinase
Ang1	Angiopoetin 1
BBB	Blood brain barrier
bFGF	Basic fibroblast growth factor
CAF	Cancer associated fibroblast
CDK4/6	Cyclin dependent kinase 4/6
COX2	Cyclooxygenase
CRISPR	Clustered regularly interspaced short palindromic repeats
CREBBP	Cyclic AMP-responsive element binding protein
CTL	Cytotoxic T-lymphocytes
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
Dll4	Delta-like ligand 4
DSB	Double-strand break
EC	Endothelial cells
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Endothelial to mesenchymal transition
eNOS	Endothelial nitric oxide synthase
FDA	Food and Drug Administration
GLUT-1	Glucose transporter type 1
HER2	Human epidermal growth factor receptor 2
HIF	Hypoxia-inducible factor
HRE	Hypoxia response elements
HUVEC	Human umbilical vein endothelial cell
IFN	Interferon
IL	Interleukin
IR	Ionizing radiation
LET	Linear energy transfer
MAPK	Mitogen-activated protein kinase

mEF	mouse embryonic fibroblasts
MDM2	Mouse double minute 2 homolog
MMP	Matrix metalloproteinase
MTF1	Metal transcription factor 1
MVD	Microvessel density
NF-kB	Nuclear factor kB
NK cells	Natural killer cells
NO	nitric oxide
NRP1	Neuropilin 1
NSCLC	Non-small cell lung cancer
NTCP	Normal tissue complication probability
PA	Plasminogen activator
PAI-1	Plasminogen activator inhibitor-1
PD-1	Programmed cell death protein 1
PDGF	Platelet derived growth factor
PD-L1	Programmed cell death protein 1 ligand 1
PFT	Pifithrin
PGI2	Prostacyclin 2
PHD	Prolyl hydroxylase domain
PI3K	Phosphoinositide 3-kinase
PlGF	Placental growth factor
RCC	Renal cell carcinoma
ROS	Reactive oxygen species
RT	Radiation therapy
SABR	Stereotactic ablative radiotherapy
SBRT	Stereotactic body radiotherapy
SCC	Squamous cell carcinoma
SCLC	Small cell lung cancer
SHH	sonic hedgehog
SMA	Smooth muscle actin
SMO	Smoothened
SSB	Single-strand break

TAM	Tumor associated macrophages
TCP	Tumor control probability
TCR	T cell receptor
TF	transcription factor
TGF	Transforming growth factor
Tie2	Angiopoetin receptor 2
TIMP	Tissue inhibitor of metalloproteinase
TKI	Tyrosine kinase inhibitor
TME	Tumor microenvironment
TSP-1	Thrombospondin-1
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VHL	Von Hippel–Lindau
WHO	World Health Organization
Wnt	Wingless

Contents

1 Introduction

1.1 Cancer overview

Cancer is a malignant neoplasm – a disease involving genomic alterations and transformation of normal cells into tumor cells that can grow uncontrollably and invade other organs through metastasis [1, 2]. The incidence of cancer is due to mutations that are acquired through incorrect DNA replication during stem cell division, through environmental factors such as air pollution, sun, lifestyle (smoking, diet) or infections (bacterial and viral) and a limited amount of inherited mutations. [3, 4]. Mutations in oncogenes and tumor suppressor genes, leading to gain of function and loss of function respectively, indicate that the disease is a multistep process**.** Most of these mutations are called passenger mutations as they do not alter the cell's functions. However, certain mutations that affect essential functions of a cell are called driver mutations and can lead to cancer.

Tumors can arise in almost every organ and are divided into two groups: benign and malignant (cancer). Compared to benign tumors, which are harmless, malignant tumors are metastatic, meaning they have the ability to disseminate from the primary site and invade a distant organ, and are major contributors to morbidity and mortality [5]. We are familiar with more than 100 types of cancer and their organ-specific subtypes [1]. Cancers obtain their names according to their origins and can exist either in solid form where it grows in an organ (e.g. carcinoma), such as lung or affect the blood system (leukemia). Carcinomas are the most common type of cancer arising from epithelial cells in respiratory system, breast, head and neck and many more. Sarcomas arise in the mesenchymal cells of muscle, bone or connective tissue. Hematopoietic cancers are blood cancers and include leukemia and lymphoma. Melanoma and cancers of neuronal system fall into category of neuroectodermal cancers.

The International Agency for Research on Cancer estimated 18.1 million new cancer cases and 9.6 million cancer deaths worldwide in 2018 using GLOBOCAN 2018. 23.4% of these cases were estimated to occur in Europe alone. For both women and men combined, lung cancer is the most common cancer type (11.6%) with 2.1 million new cancer cases and the leading cause of cancer related deaths (18.4%) [6].

In 2000 and 2011, Douglas Hanahan and Robert Weinberg published two separate articles, providing a list of principles that are acquired by tumors in the process of carcinogenesis with the latter article having an updated version with a total of 10 hallmarks [1, 7]. These hallmarks have been used in basic and translational research as a basis for a better understanding of cancer and for improving the treatment strategies. Normal cells are able to regulate stimulatory and inhibitory signals and therefore maintain homeostasis. *Sustaining proliferative signaling* is the most important feature of cancer cells where they can deregulate the homeostasis by increasing the production of growth factors from themselves or the surrounding cells. Moreover, downstream signaling pathways can also be constitutively activated independent of growth factors. Additionally, cancer cells are able to *evade growth suppressors* by acquiring mutations in different tumor suppressor genes. Apoptosis is one form of a programmed cell death that is triggered in response to various stress signals. In cancer cells, due to increased oncogenic signaling, several imbalances occur in apoptosis inducing pathways and therefore can *resist cell death*. In order to create a tumor, cancer cells can *enable replicative immortality* by extending the telomeres using active telomerase. Cancer cells *induce angiogenesis* by increasing the expression of several pro-angiogenic factors, such as vascular endothelial growth factor (VEGF). This in turn leads to new vessel formation and therefore cancer cells can supply themselves with oxygen and nutrients. By modifying the proteins involved in cell-cell and cell-extracellular matrix (ECM) adhesion, cancer cells are able to *activate invasion and metastasis* related pathways. Usually, cancer cells acquire mutations in genes involved in detection and repair of DNA damage. These mutations result in *genomic instability* and growth advantage. Pro-tumorigenic proteins, such as angiogenic and growth factors in the tumor microenvironment, are involved in *tumor-promoting inflammation*. *Deregulating cellular energetics* from oxidative phosphorylation to aerobic glycolysis supplies tumor cells with glycolytic intermediates for different metabolic pathways. Cancer cells are able to *avoid immune destruction* by upregulating immune inhibitory surface molecules or recruiting immune suppressive cells. Figure 1 shows a short summary of these hallmarks and treatment strategies to target each of the hallmarks.

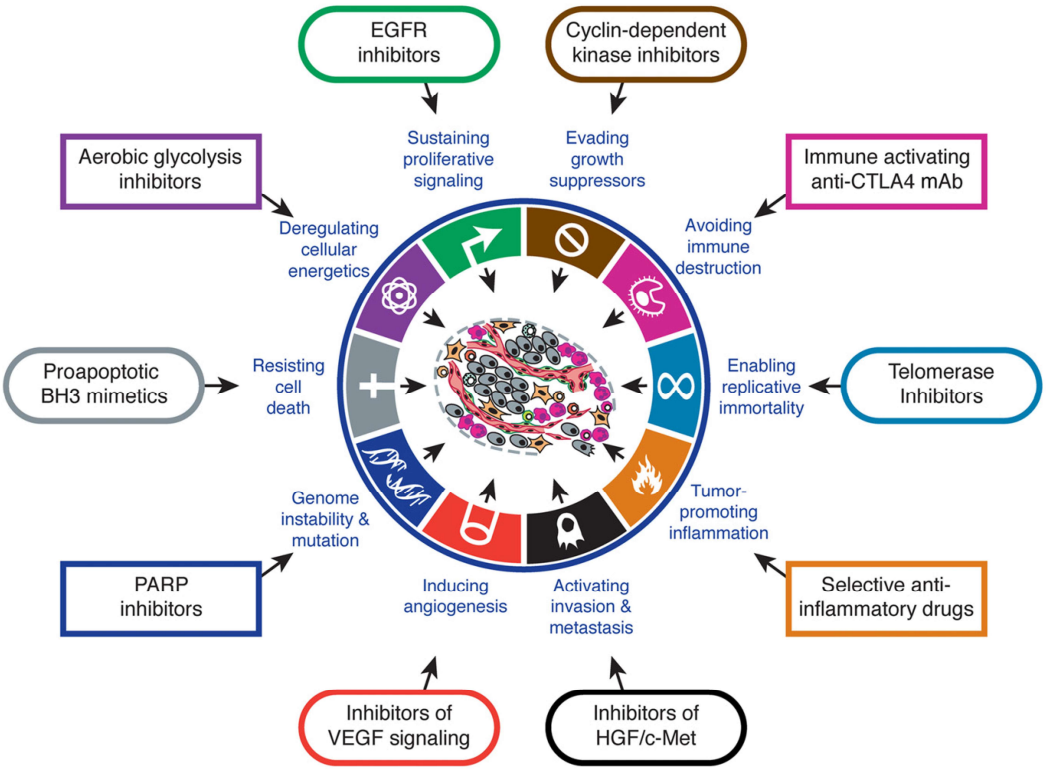

Figure 1. Hallmarks of cancer and their treatment strategies. Cancer cells possess several hallmarks in order to grow and survive. Treatments strategies of cancer is based on these hallmarks and the targeting each of these with the appropriate drugs. Figure adapted from [7].

1.2 Cancer treatments

1.2.1 Radiotherapy

X-rays were discovered in 1895 by Roentgen and shortly after radiation was used to treat cancer for the first time [8]. Over the years, there were major developments that lead to a drastic increase in benefits [9, 10]. These developments include advances in technology and a better understanding of molecular pathways involved in treatment response [11-16].

Ionizing radiation (IR) is a process where the absorption of radiation leads to ejection of one or more electrons from an atom or a molecule. These high energy, accelerated electrons have a high probability to interact with matter, induce downstream

ionizations (“electron splash” by secondary electrons) thereby leading to breakage of chemical bonds. Radiation can either be directly or indirectly ionizing. Charged particles (electrons and protons) are directly ionizing, meaning each particle has enough energy to directly disrupt an atom. Electromagnetic radiations (x-rays), on the other hand, are indirectly ionizing and can only produce damage when they are absorbed in a material, can give up their energy to the (ejected) electron of a molecule, generating an electron splash and eventually producing free radicals. For example, the hydroxyl radical is highly reactive and can diffuse to DNA to induce critical damage. In order to be biologically active and considered ionizing, the electromagnetic radiations should have an energy of at least 124eV. A dose of 1Gy (1J/kg) can induce 40 double-strand breaks (DSB), 1000 single-strand breaks (SSB) and 1000 base pair damages [17].

Radiation therapy (RT) is an essential and effective method for cancer treatment with survival and palliative benefits for patients and is widely used for management of cancer [18-22]. The aim of RT is to distribute the optimal dose to the tumor in order to kill the cancer cells and to shrink the tumor while sparing the normal tissue. Usually, RT is combined with surgery and chemotherapy. In case of inoperable or recurrent tumors, RT is the choice of treatment [23]. Approximately 50% of all cancer patients are treated with RT [24, 25] with curative or palliative aim [23, 24, 26-29]. Despite a remarkable progress in RT, there is still a need to improve the curative rate.

RT is typically applied in multiple fractions over several weeks in order to increase the therapeutic index [27]. Different types of radiation sources, such as photons or protons, can be used to improve the efficacy of the treatment, to effectively kill tumor cells while sparing the normal tissue (biological effective dose) [10, 30]. Radiation can be delivered either by external beam radiation therapy or with radioactive material placed close to cancer cells (brachytherapy) inside the body. Different types of radiation have different linear energy transfer (LET). LET is the number of ionizations per unit distance caused by radiation as it crosses the living tissue. LET, fractionation, dose rate, radiosensitivity of tissues and total dose are the determinants of biological effectiveness [31]. X-rays, gamma rays and beta particles are all low LET radiations and deposit a small amount of energy per ionizing event. Negatively charged (electrons) or positively charged (protons, heavy ions) particles deposit more energy on the target area (Bragg peak) and can cause more relevant biological effects.

Nevertheless, tumors can resist radiation induced damage due to intrinsic or acquired resistance to therapy, tumor burden and repopulation during the course of treatment [32]. Either alone or in combination with surgery and/or chemotherapy, high doses of ionizing radiation can achieve almost 100% of tumor control probability (TCP) [11]. However, at the same time, healthy tissue is also exposed to radiation, which leads to increased normal tissue complication probability (NTCP) and limited treatment success rates. Therefore, the major goal of experimental research in radiation oncology is to extend the therapeutic window by reducing the normal tissue toxicity and at the same time increasing tumor control [23, 33].

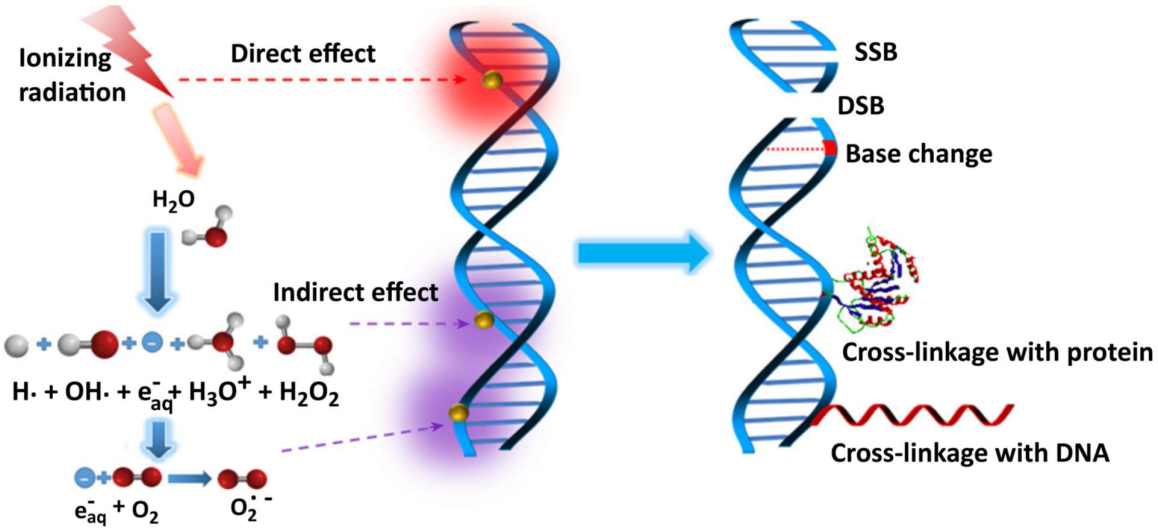

Figure 2. Mechanisms of ionizing radiation (IR). IR can damage the DNA either directly or indirectly by generating reactive oxygen species (ROS) from water molecules inside the cell. Types of DNA damage: single-strand break (SSB), double-strand break (DSB), cross-linking, base damage. Figure adapted from [34].

1.2.1.1 The 5 R's

The 5 R's of radiobiology have played an important role not only in the development of safe, precise and effective treatment regimens, but also in our understanding of biological effects of radiation. They have also been involved in characterization of tumor and normal tissue response to fractionated RT. The following list of 5 R's [35] summarizes our knowledge in improving local tumor control and reducing normal tissue toxicity:

Repopulation: each fraction of RT decreases the number of surviving tumor cells. However, the surviving cells can grow and repopulate between the fractions. Repopulation is evident three to four weeks after the start of conventional RT and is one of the causes to treatment resistance.

Repair: IR ejects electrons from molecules within the cells, leading to their collision with DNA and further ionizations. This can induce single-strand or double-strand breaks on the DNA (SSB and DSB, resp.), the latter particularly difficult to repair.

Redistribution: radiosensitivity of cells varies according to the cell cycle. Cells in S phase are more resistant to RT than cells in late G2/M phase. Moreover, cells are able to repair damage differently in the various phases of the cell cycle. The surviving cells after RT redistribute to next phases of the cell cycle.

Reoxygenation: tumors contain a mixture of normoxic and hypoxic areas. Reoxygenation refers to the process where hypoxic cells become oxygenated after RT. Hypoxic cells are less sensitive to RT, which leads to treatment failure. Normoxic cells, on the other hand, are more sensitive to RT and the RT-induced damage is more permanent.

Radiosensitivity: intrinsic radiosensitivity is genetically determined and varies between tissues depending on the proliferation, function and ability of cells to undergo a mode of programmed cell death. Susceptibility to e.g. apoptosis contributes to radiosensitivity and may explain heterogeneity within and between cancer types.

1.2.2 Chemotherapy

Chemotherapy is a systemically-applied therapy, meaning besides killing cancer cells, chemotherapy will also affect normal tissues and will therefore lead to high levels of normal tissue toxicities and treatment side effects. Nevertheless, chemotherapy is widely used in the clinics in combination with surgery or radiotherapy. Based on their mechanism of action and target, chemotherapeutic drugs are divided into the following groups:

Alkylating agents: Alkylating agents are reactive compounds with the ability to modify the structure of different molecules (such as DNA or proteins) by transferring alkyl

carbon groups onto them. These compounds react with nitrogen or oxygen atoms of DNA and generate irreversible covalent bonds, which later disrupts the DNA processes (e.g. DNA replication) [36-38].

Anti-metabolites: even though anti-metabolites may have different actions that either lead to cytotoxicity or radio-sensitization, they all target DNA replication. Based on this, anti-metabolites can be divided into two groups: a) agents inhibiting deoxyribonucleotides for replication (e.g. fluorouracil) and b) agents becoming false substrates for DNA polymerases (e.g. gemcitabine) [39].

Topoisomerase inhibitors: Topoisomerases are enzymes essential for transcription and replication and can regulate DNA supercoiling and entanglements by different mechanisms. Topoisomerase inhibitors are powerful and selective drugs used as anticancer therapy. Inhibition of topoisomerases leads to formation of abnormal DNA structures that results in cell cycle arrest [40].

Microtubule interfering agents: drugs in this category act on microtubules, either stabilizing (e.g. paclitaxel) or disrupting (e.g. vincristine) the microtubule polymers. Impaired microtubule structure and function leads to cell cycle arrest and cell death [41].

1.2.3 Targeted therapy

The aim of targeted therapy is to inhibit or modify specific signaling pathway and proteins involved in tumor growth. Targeted therapy is divided into two categories: 1) small molecules that can pass through the cell membrane and interfere with targets inside the cells (tyrosine kinase inhibitors: imatinib, gefitinib), 2) monoclonal antibodies that are not able to enter the cells and can target specific proteins on the cell surface (bevacizumab, cetuximab). Monoclonal antibodies can also be delivered as antibody-drug conjugates (ADCs). In this case, when an antibody binds to its target on cancer cells, the conjugated drug is incorporated and released into the cancer cell [42-44]. It is important to identify specific molecular markers expressed by cancer cells as targets for either of these categories in order to increase the therapeutic window so that patients will benefit from this type of therapy. Overall, targeted therapy is associated with improved effect on the tumor level and decreased toxicity.

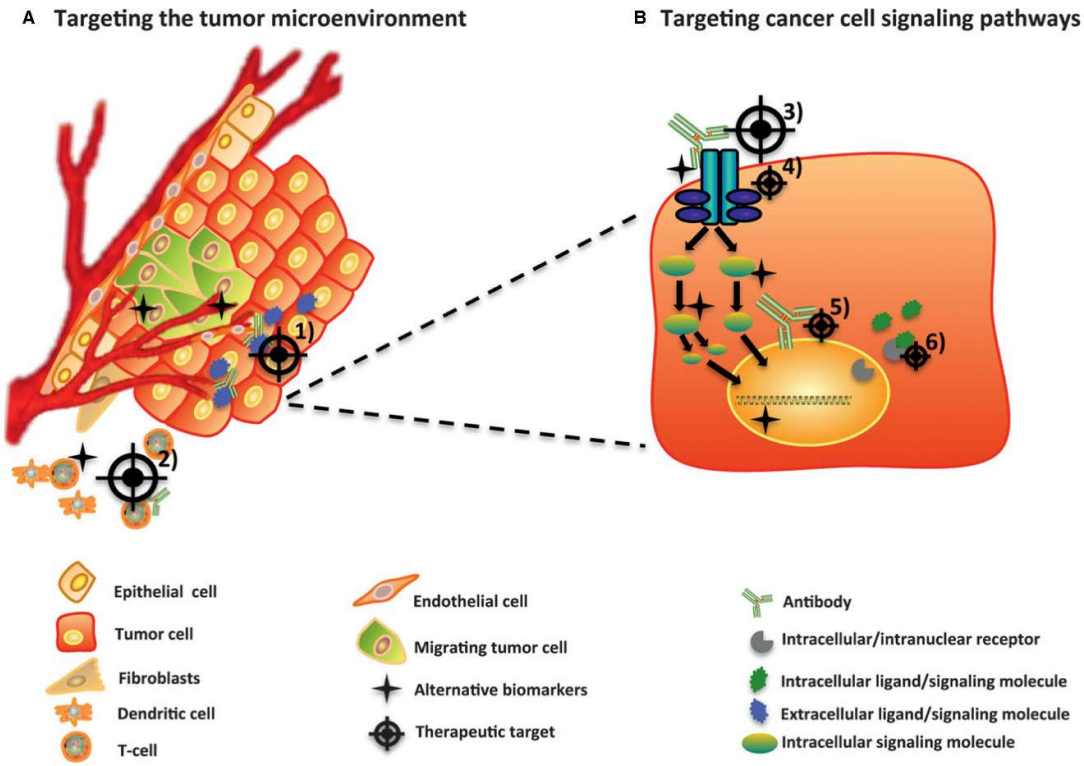

Figure 3. A model for use of targeted therapy. Targeted therapy can either focus on targets in the tumor microenvironment or on targets within the cancer cells. Targeted therapies directed against targets in the tumor microenvironment include blood vessels (1) and the immune system (2). Signaling pathways within the cancer cells can be targeted by antibodies against extracellular domain of transmembrane receptors (3) or using inhibitors against their intracellular tyrosine kinase domains (4). Other targets include proteins involved in nuclear signaling (5 and 6). Figure adapted from [43].

1.2.4 Immunotherapy

Over the last few years, immunotherapy has become of great interest due to its ability to treat certain cancer forms either through immune activation or suppression. Immunotherapy is not as widely used as other therapies mentioned here. However, some immunotherapies are approved for cancer treatment and several immunotherapies are being investigated in clinical trials. Immunotherapeutic drugs are categorized into two groups:

1) drugs blocking negative regulatory signals and thereby inhibiting immune evasion: Cytotoxic T lymphocyte-associated protein 4 (CTLA-4) is expressed on T cells in very low levels, but is upregulated upon T cell receptor (TCR) activation and peptide complex formation and acts as a negative regulatory receptor [45]. CTLA-4 competes with its homolog CD28, a costimulatory receptor on T cells, for the same ligands

(CD80 and CD86) [46]. Anti-CTLA-4 monoclonal antibodies have shown incredible clinical results with regard to tumor regression in advanced melanoma. These antibodies disrupt CD28 activation and deplete regulatory T cells (Tregs) [47].

Programmed cell death protein 1 (PD-1) is a co-inhibitory receptor with high expression on activated T cells, B lymphocytes and natural killer (NK) cells [48]. Its expression is induced upon TCR-peptide complex formation in the effector phase of the immune response. It has two ligands (PD-L1 and PD-L2) that are expressed in low levels in healthy tissues. Binding of PD-1 to PD-L1 on tumor cells may provide the tumor cell with resistance to cytotoxic T-lymphocytes (CTLs) and apoptosis induced by the Fas pathway. Increased PD-L1 expression has been reported in melanoma, head and neck cancer, non-small cell lung cancer (NSCLC) and breast cancer subsequently leading to increased benefit with PD-1 axis blockers [47].

2) drugs stimulating immunogenic pathways: Vaccines enhancing the antigen presentation, oncolytic viruses and exogenous cytokines are all part of immune-stimulatory therapies. Activation of phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) increase PD-L1 expression in preclinical studies. PD-L1 expression is also increased in response to proinflammatory cytokines such as interferon gamma (IFN-γ) and IL-4. Even though many immune-stimulatory drugs targeting immune checkpoint pathways have shown promising clinical results, a majority of patients do not benefit from these drugs due to heterogeneous immunogenicity across tumors among other reasons [47, 49].

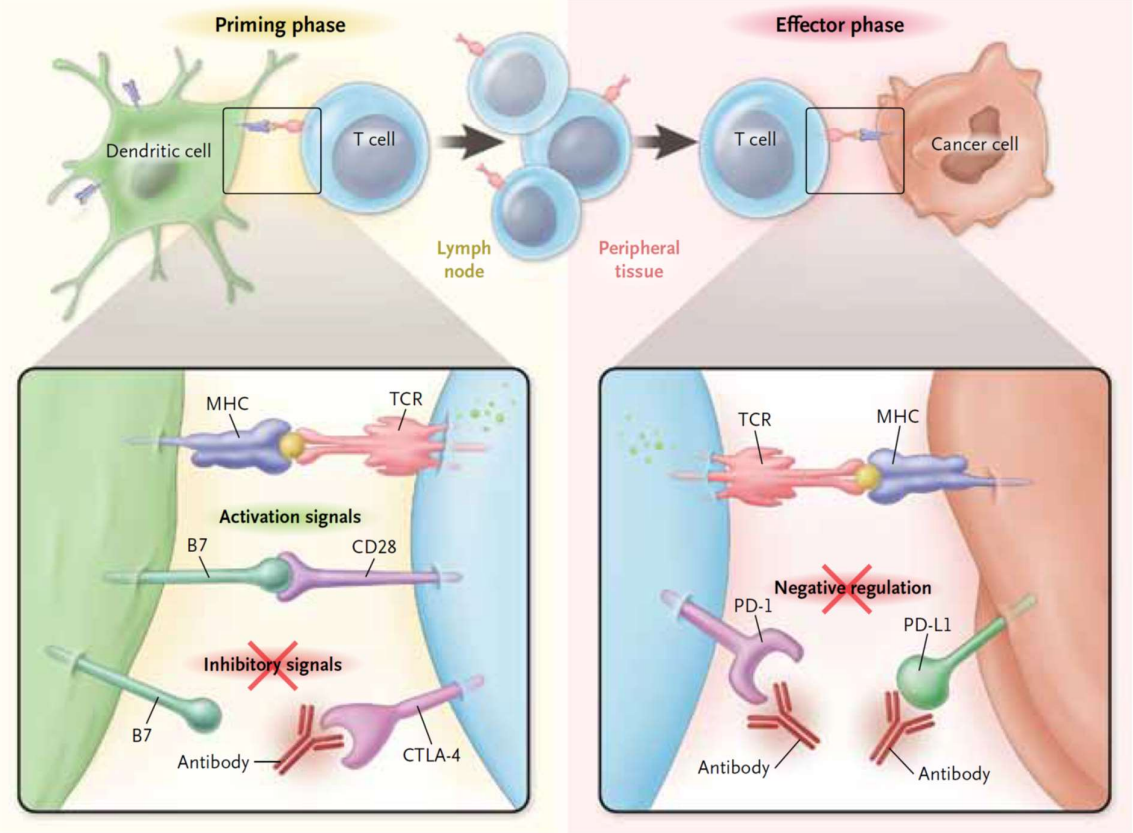

Figure 4. Blocking PD-1, PD-L1 or CTLA-4 in tumor immunotherapy. Recognition of antigens by T-cell receptor activates T cells. A second signal through costimulatory molecules is required for a full activation. These molecules can either lead to positive or negative T cell regulation depending on where they bind. Figure adapted from [50].

Most of the cell lines used in this project are either medulloblastoma (section 1.3) or lung cancer (section 1.4) cell lines. Both of these tumor entities are treated with RT in the clinics and show increased placental growth factor (PlGF (see section 1.5.4)) levels and therefore are the main focus of this thesis. Both tumor entities are introduced separately.

1.3 Medulloblastoma

In 1925, doctors Harvey Cushing and Percival Bailey introduced the term "medulloblastoma" for the very first time. Patients with this tumor were usually subjected to surgery, which was the only treatment for these patients at that time and presented dismal outcomes [51, 52]. The survival rate in medulloblastoma patients

increased significantly after the introduction of craniospinal irradiation by Paterson and Farr in 1953, even though young patients suffered from severe side effects. In order to improve this, surgery and radiation therapy were combined with chemotherapy starting in the 1970s [51].

Currently, medulloblastoma is one of the most frequent malignant childhood brain cancers, responsible for 8-10% of all childhood brain tumors. It arises in the cerebellum and is classified as a grade IV tumor by World Health Organization (WHO). Each year 1.5-2 cases/100 000 people are reported, most cases occurring between 5-7 years of age and affects boys almost two times more than girls [52]. Several studies have profiled the genetic mutations of medulloblastoma, identifying mutated genes and correlating them to the specific molecular subgroups of medulloblastoma [53-56]. The major problems of medulloblastoma are 1) the lack of effective drugs due to few targets, 2) the heterogeneity of the disease within a subgroup and 3) resistance to therapies due to acquired genomic alterations during the disease [57].

Medulloblastomas are divided into four molecular subgroups with distinct transcription profiles and clinical outcomes: wingless (Wnt), sonic hedgehog (SHH), Group 3, and Group 4. Some evidence suggest that prognosis is associated with the individual subgroup and their respective gene expression [53, 58].

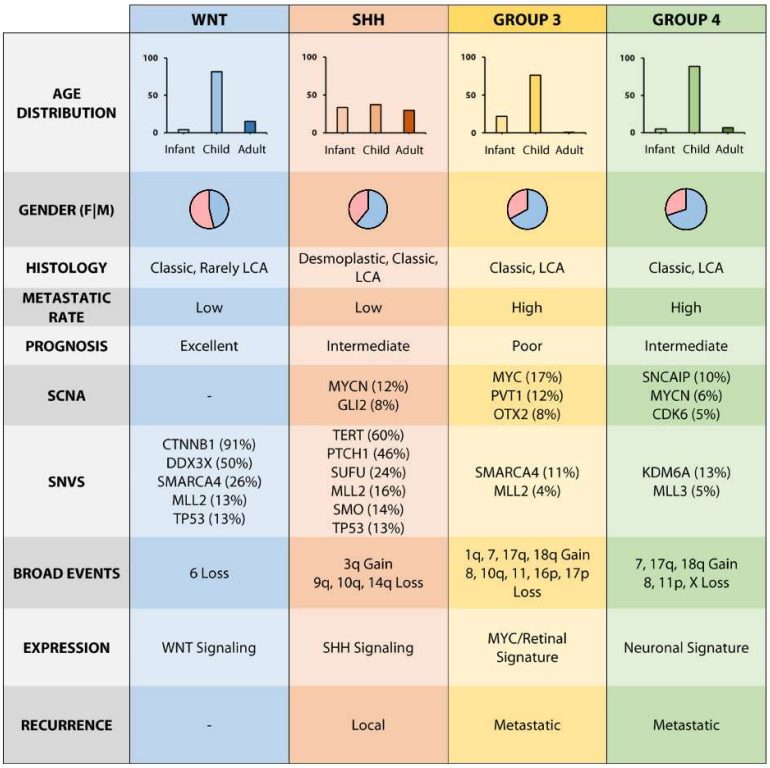

Figure 5. Medulloblastoma subgroups and their characteristics. Medulloblastoma has four different subtypes with specific genetic expression, prognosis and survival rates. Figure adapted from [59].

1.3.1 Wingless

This molecular subgroup is the least common, responsible for approximately 11% of medulloblastoma and affects both sexes to the same extent. Usually patients are 6-10 years at the time of diagnosis and have a positive prognosis with 5-year survival almost 90% [57]. Tumors show activated WNT signaling pathway with monosomy 6 and point mutation in β-catenin (CTNNB1) [55]. WNT signaling is involved in variety of processes, such as angiogenesis in CNS, bone formation and regulation of other signaling pathways. Therefore, targeting the interaction between CTNNB1 and CREB binding protein (CREBBP) is being evaluated. It is worth noting that targeting WNT pathway might also lead to resistant tumors, as WNT signaling might improve the effect of chemotherapy [57].

1.3.2 Sonic Hedgehog

The Sonic hedgehog (SHH) subgroup affects mostly the youngest and the oldest patients with bimodal distribution. This subgroup is named after the most dominating signaling pathway in these tumors, namely SHH and accounts for 25% of all medulloblastoma cases [57]. The survival rate in this group largely depends on the TP53 status of the tumor, with better 5-year survival rate for TP53 wild-type tumors compared to TP53 mutated tumors. TP53 mutated tumors are resistant to therapies and have increased MYCN amplification [60]. Other mutations in this subgroup also include the SHH receptor smoothened (SMO) [54]. Additionally, SHH medulloblastoma shows different gene expression profiles depending on the age of the patients [61]. The best way to treat this subgroup of medulloblastoma is to use SMO inhibitors, such as Vismodegib, as long as the mutations observed are upstream of the receptor. However, this type of treatment is most favorable for adult patients [57].

1.3.3 Group 3

Expression of MYC, deletion in chromosome 11 and isochromosome 17q among other genetic abberations are signatures of Group 3 medulloblastomas [53, 56, 62]. 25% of all cases belong to this subgroup and it has the worst prognosis out of all groups with 5-year survival rate of 20% [56]. 50% of Group 3 tumors are mostly diagnosed at metastatic state and are constantly in focus for potential therapies because of the disease outcome [63, 64]. For example, cycline-dependent kinase 4/6 (CDK4/6) inhibitor Palbociclib, approved for treatment of breast cancer, is in clinical trial for medulloblastoma patients since MYC overexpressing tumors rely on CDK4/6 for their proliferation and migration. Compared to WNT and SHH groups, p53 mutations are absent in this subgroup [60].

1.3.4 Group 4

This is the most common subgroup with 35% of all cases, although not much is known about Group 4 medulloblastomas. It consists of chromosomal copy number variations and i17q. However, compared to Group 3, patients in Group 4 benefit from i17q or loss of chromosome 11. Overall, depending on the risk group, patients in this subgroup

have great prognosis that can go up to 95% [65]. Some research groups suggest that inflexible epigenetic regulations could be a signature of this group [66, 67].

1.3.5 Treatment of medulloblastoma

Treatment of medulloblastoma is based on the risk criteria, meaning whether it is a standard-risk (SR) or a high-risk (HR) patient. Children over 3 years of age without a metastatic disease and a tumor less than 1.5cm^2 are considered as SR, whereas the rest are HR. HR patients usually present a metastatic disease and have poor prognosis [68]. A combined treatment modality is used for treatment of children older than 3 years, which is surgical resection followed by chemotherapy and craniospinal RT [69]. Usually, patients receive combined chemotherapy of cyclophosphamide, cisplatin and vincristine [57]. Younger children are subjected to surgery and high-dose chemotherapy without irradiation, which can give up to 75% 5-year survival depending on the subgroup [69, 70].

However, the treatments have severe side effects including cognitive and endocrine dysfuntion, hearing loss and increased cancer risk [71, 72]. Although medulloblastomas are radiation sensitive, this type of treatment leads to loss of up to 40 IQ points [73].

There is an urgent need for alternative treatment options. Lately, the focus has been on targeted therapy using drugs against molecules that have a driving role in medulloblastoma. One of these targets is Notch signaling pathway, which is involved in survival and proliferation of neuronal cells [74]. Other factors involved in medulloblastoma formation are VEGF, platelet-derived growth factor (PDGF) and cyclooxygenase 2 (COX2) among others revealed by preclinical research [25]. Clinical trials are performed combining targeted therapy with chemotherapy or anti-angiogenic agents [69].

1.4 Lung cancer

The most common cause of cancer-related deaths in the world is due to lung cancer [6]. 85–90% of lung cancers are non-small-cell lung cancer (NSCLC) with 5-year survival rate approximately 16%, while the rest fall into small-cell lung cancer (SCLC) [75]. NSCLC is characterized into subtypes based on their histology and genotype, with

distinctive features and prognoses. There are two subtypes of NSCLC: adenocarcinoma arising in the distal airways and squamous cell carcinoma (SCC) more frequent in proximal airways [76]. The two subtypes are distinguished by immunostaining for cytokeratin 5 and 6 or specific transcription factors [76, 77].

Radiotherapy plays an important role in treatment of both early-stage and advanced stage NSCLC and SCLC with curative or palliative intent. Patients with inoperable early-stage NSCLC are treated with stereotactic ablative radiotherapy (SABR) with curative intent using several modern imaging technologies [78]. Post-SABR failures include local recurrence, lymph node failures and development of distant disease. Further therapies for these patients are usually radiotherapy and chemotherapy [79]. Locally advanced NSCLC is still difficult to treat showing local and distant failures after chemoradiotherapy [80]. However, the recommended treatment for these patients is still conventional fractionated chemoradiotherapy [78]. SABR (1-5 fractions) combined with chemotherapy is also used as a promising treatment method for early-stage SCLC showing good local control rate [81].

Green and colleagues demonstrated that lung tumors do not only differ in histology, but also in their response to different drugs by showing that lung SCC was more sensitive to nitrogen mustard, while SCLC responded best to cyclophosphamide [82]. In 2002, a randomized clinical trial assessed the efficacy of frequently used chemotherapeutic treatments (cisplatin plus either paclitaxel, gemcitabine or docetaxel, and carboplatin and paclitaxel) showing no survival advantage with any of the treatment regimens compared to each other [83].

Lung cancer was accepted to be a heterogeneous disease already in the 1950s. However, treatment options remained unchanged until 2004 when it was accepted that mutational status of the disease plays an important role in treatment response. For example, epidermal growth factor receptor (EGFR) mutation and the response to gefitinib lead to identification of a specific group of patients [84, 85]. Additionally, high response rates and high disease-control rates are achieved with erlotinib, gefitinib, and afatinib in patients with EGFR mutations, and with crizotinib and ceritinib in patients with anaplastic lymphoma kinase (ALK) translocations [85]. Although patients with ALK translocations respond >70% to crizotinib treatment, most of them relapse within the first year of treatment. Erlotinib also shows similar responses in patients EGFR mutations, nevertheless the survival rates are low [85]. Resistance to these treatments include mutations, gene amplifications or alternative

splicing of the target gene or activation of other oncogenic pathways (e.g. BRAF mutation, HER2 amplification etc.). Furthermore, insufficient exposure to the drug due to hindered interactions and pharmacokinetic differences can also contribute to resistance mechanisms [86, 87].

Resistance mechanisms are not exclusive and several resistance mechanisms can exist in one tumor [87]. To overcome this hurdle, multiple therapies are used in combination. Nevertheless, the diversity of pathways interacting and the sensitivity of these pathways to different drugs make this task rather difficult.

By producing tumor antigens, RT can also stimulate the immune system, promote anti-tumor T-cell response and possibly lead to abscopal effects causing regression of other lesions. There is an ongoing research about the optimal dose of RT and its fractionation and the combination with immunotherapies [88]. Anti-tumor immune responses to treat several types of cancer, including NSCLC, have been of interest for many years [89]. However, there have been limitations due to immune-evasion mechanisms in the tumor, such as secretion of immunosuppressive cytokines or upregulation of immune-checkpoint proteins. Additionally, activating mutations in EGFR pathway have shown to increase PD-L1 expression [90]. Tumor cells express PD-L1 on the cell surface to inhibit eradication by T cells that express PD-1. Clinical trials blocking PD-1–PD-L1 interaction have shown promising results in a subgroup of patients [85]. Nivolumab, a PD-1 antibody approved for patients with metastatic lung SCC, showed prolonged overall survival in previously treated patients compared to docetaxel treated patients in a phase III trial [85]. At the moment, there is a need to define NSCLC subsets based on the immune response and further investigations on immune-evasion mechanisms could offer treatment options to promote better tumor targeting.

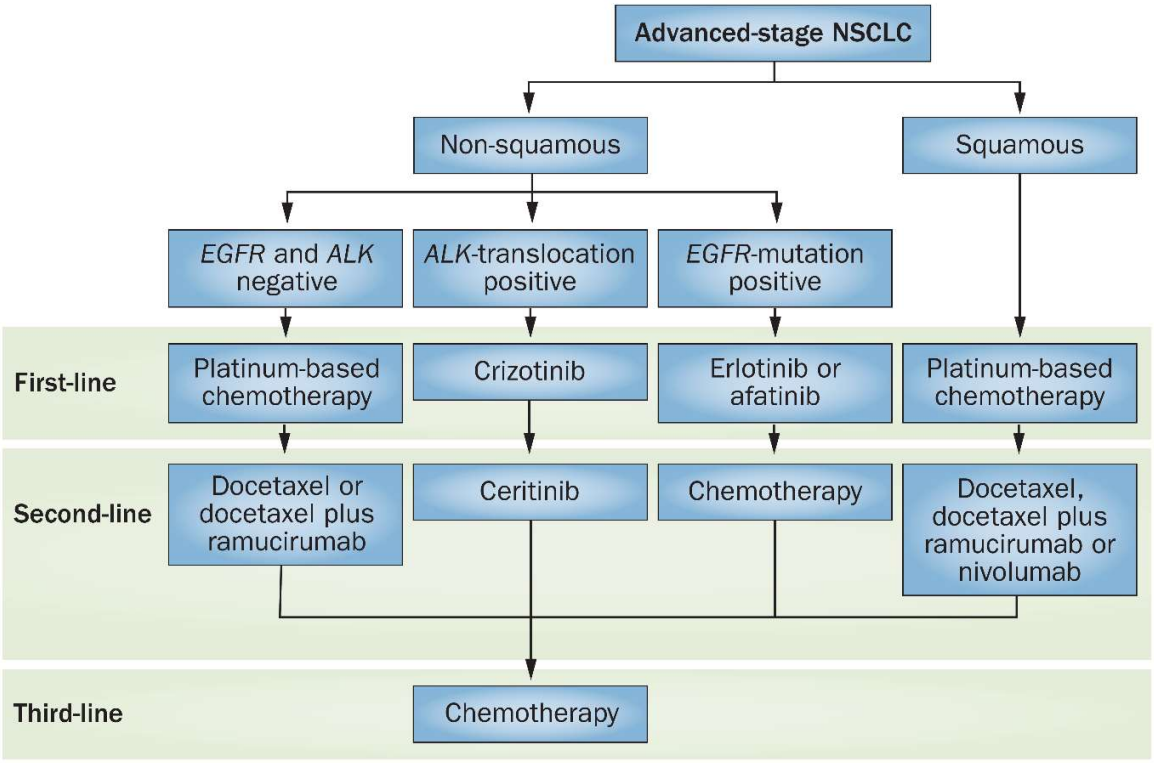

Figure 6. Summary of treatment options for advanced stage NSCLC. Depending on the genetic expression, different subtypes of lung cancer are treated accordingly. Figure adapted from [85].

1.5 Angiogenesis

1.5.1 Normal angiogenesis

Blood vessels are necessary for supplying oxygen and nutrients, and removing the metabolic waste in living organisms. During embryonic development, the vascular network develops through vasculogenesis, a process where vessels are formed de novo from angioblasts. Angioblasts proliferate and form a network of vessels which later serves as a support. These network of vessels are then modified by the sprouting and branching of vessels leading to angiogenesis, the formation of new vessels from preexisting ones [91, 92]. Normal angiogenesis has a central role in wound healing, ovarian cycle, organ perfusion and establishing an adequate vessel support for growing organs during embryonic development [93]. Under physiological conditions, blood vessels remain dormant and do not form new vessels. However, irregularities in

angiogenesis are linked to many disorders, including ocular neovascularization and cancer [92, 94, 95].

Briefly, the following steps are involved in angiogenesis: 1) pericytes are detached from the blood vessel and different proteases are produced to degrade the ECM and the basement membrane [96-98], 2) endothelial cells (ECs) proliferate and migrate to the perivascular area, 3) and form tube-like structures in monolayer from primary sprouts, 4) new basement membrane is produced, and 5) pericytes or smooth muscle cells are recruited to the surface of the endothelium supporting vessel maturation and preventing vascular leakage by covering the vessels leading to stabilized blood vessels [92, 95, 99]. Pericytes are recruited to the area by PDGF-B secreted by ECs. Another factor required for the maturation process is angiotensin 1 (Ang1) which can activate Tie2 receptor on ECs and activate AKT pathway, leading to survival in ECs [100].

VE-cadherin, among other adhesion molecules, is an important protein involved in supporting the cell junctions between ECs and decreasing vascular permeability by binding to each other on neighboring ECs [101].

Under normal conditions, angiogenesis is a tightly regulated process with several growth factors involved [99]. Growth factors including VEGF, stimulate ECs and lead to their migration. ECs located in the beginning of the vessels are called tip cells. They are invasive and can initiate sprouting, whereas following stalk cells support the structure and function of the newly built vessels and are mainly regulated by the Notch pathway. VEGF secretion activates VEGFR 2 and Neuropilin 1 (NRP1), which in turn increase the expression of the Notch ligand Delta-like 4 (Dll4) [102]. Upon binding to its receptor on ECs, Dll4 leads to the release of the intracellular domain of Notch1. This domain acts as a transcription factor and controls the VEGF signaling by inhibiting the gene expression involved in VEGF responses, such as VEGFR2 and 3, and NRP1 [103, 104]. VEGFR1 on the other hand, is increased in response to Notch1 signaling. Even though VEGFR1 has stronger affinity to VEGF, it has weaker kinase activity. Therefore, it can bind VEGF and reduce its activity through VEGFR2 [105]. Another ligand of Notch1 is JAGGED1 (JAG1) expressed on stalk cells, negatively regulating Dll4-Notch1 signaling [106].

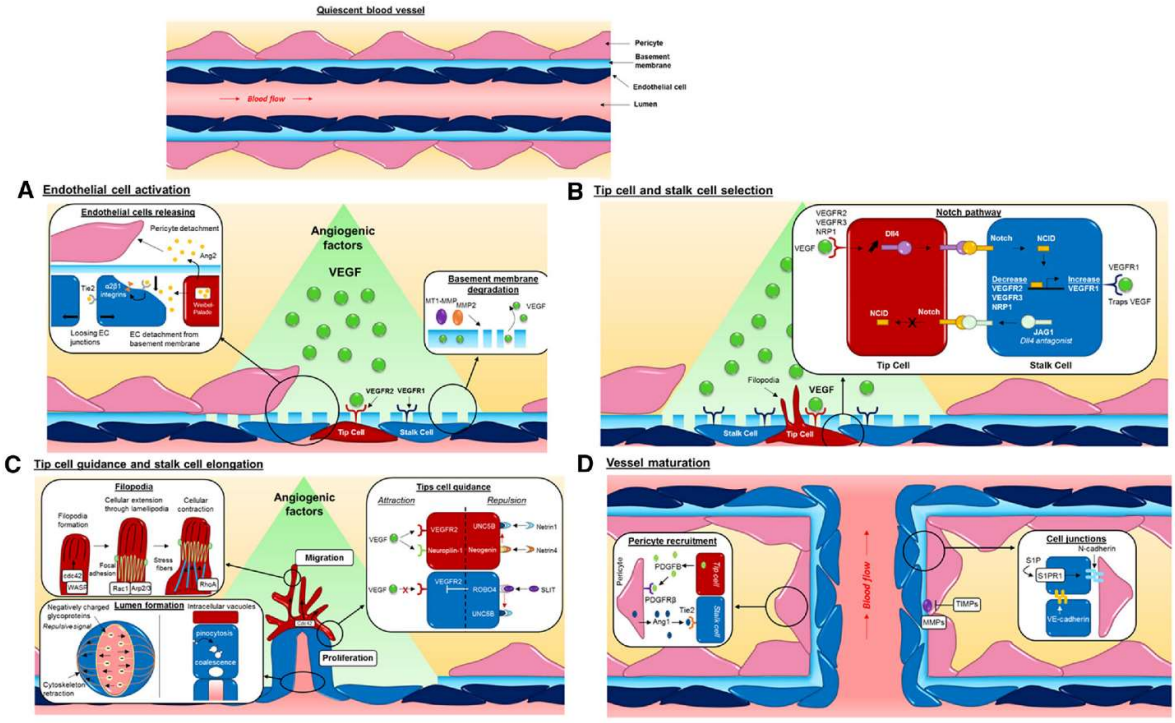

Figure 7. Normal angiogenesis and its stages. A-D) blood vessels destabilization, detachment of pericytes from basal membrane and ECM degradation, EC stimulation by growth factors (e.g. VEGF), lumen formation, blood vessel maturation by pericyte recruitment. Figure adapted from [92].

1.5.2 Tumor-induced angiogenesis

Tumor cells are highly proliferative host-derived cells that have lost the capacity to regulate growth. Although there are many differences between a normal and a tumor cell, some of the characteristics are similar. One of these characteristics is the need for a sufficient oxygen and nutrient supply and effective ways to eliminate waste in order to maintain survival. In order to support their growth, they need to develop new blood vessels very fast. To satisfy these demands, tumor cells can induce oxygen and nutrient supply from the preexisting vessels in a similar way to normal angiogenesis [107]. However, these vessels are most of the time immature and lack functionality observed in normal tissue [108]. VEGF, transforming growth factor (TGF-β), angiopoietins, PDGF-B and other protein families have been associated with imperfect vessels in tumors [109]. The improper vessels without pericyte coverage can lead to several consequences, such as hypoxia, increased metastasis and decreased activity of immune cells [99, 108]. Compared to healthy tissue vasculature, the tumor vessels are

twisted, enlarged, damaged, leaky and disordered, which all in turn lead to hypoxia and increased permeability. The walls of these vessels can consist of both ECs and tumor cells [110]. The connection between pericytes and ECs are compromised due to degraded VE-cadherin and therefore the regulation of the vessel permeability is impaired [101]. Another important element of blood vessels is the basal membrane. There are differences in basal membrane structure between healthy and tumor associated vasculature regarding the coverage by smooth muscle actin (SMA), CD31 and type IV collagen. Other structural irregularities include uneven vessel thickness and punctures [92, 111]. Even though the vessels show many abnormalities, they are still able to support tumor cells using mechanisms and molecules involved in normal angiogenesis [112].

1.5.2.1 Tumor vasculature

Surrounding healthy tissue compresses tumor cells growing uncontrollably which leads to suppression of tumor vessels. In addition, decreased perfusion due to leaky vessels causes increased pressure in the interstitial space. These events also decrease the supply of nutrients and oxygen to tumor cells subsequently leading to hypoxic tumors and resistance [113]. As mentioned above, one of the main regulators of angiogenesis in tumors is hypoxia. When oxygen is present, hypoxia inducible factors (HIFs) are hydrolyzed by prolyl hydroxylase domain protein 2 (PHD2), ubiquitylated by Von Hippel–Lindau (VHL) complex and degraded by the proteasome [92, 114]. During hypoxia however, PHD2 is inactive, which leads to HIF1-α stabilization and binding to hypoxia response elements (HREs) of the target genes such as VEGF, glucose transporter type -1 (GLUT-1) and TGF-β [115, 116]. In addition, hypoxia causes activation of oncogenes and increases the metastatic ability of tumor cells by supporting epithelial to mesenchymal transition (EMT) [92].

In order to build a vascular network, tumor cells use either sprouting or intussusceptive angiogenesis [117]. However, there is an advantage of intussusceptive angiogenesis: vessel formation is faster with lower metabolic requirements [118]. Intussusceptive angiogenesis occurs through formation of new bridges from preexisting vessels and is performed in following steps: 1) using collagen bundles, ECs bind to each other and build bridges in the lumen, 2) these collagen bundles migrate

into the lumen through degraded basal membrane, 3) a bridge is formed by connection of collagen bundles to the of the lumen, 4) the bridge matures by recruiting mural cells, such as pericytes [92].

Chaotic, leaky and tortuous tumor vasculature has negative effects on various cancer therapies. For example, in presence of oxygen, radiotherapy causes the generation of reactive oxygen species (ROS), DNA damage and subsequently cell death [119]. Thus, the hypoxic areas of the tumor are less sensitive to irradiation [120]. Additionally, hypoxia can also interfere with the delivery and effect of chemotherapeutic drugs. Due to collapsed vessel, various drugs might not reach all areas in the tumor and therefore have reduced effect [113] as many agents, such as doxorubicin need oxygen to effectively lead to cell death [92].
Decreased perfusion is also reducing the efficiency of brachytherapy where tumors are irradiated by using a 188-Rhenium labeled antibody [121]. These antibodies require oxygen and an even distribution for their action. The similar reduction in therapy effect is also observed with immunotherapies where immune cells have difficulties to reach the tumor because of immature blood vessels [122].
Oxygen level, which can be sensed by different types of cells, is an important regulator of angiogenesis [123]. Solid tumors have a necrotic region in the center because of low oxygen level [99]. The family of HIF-related transcription factors plays a central role in controlling the cellular responses to changes in oxygen level. The three isoforms of HIF-α (HIF-1–3), which are only stabilized during hypoxia, can heterodimerize with the constantly expressed HIF-1β and induce the expression of several genes [124]. Oxygen is not only used by healthy cells, but also by tumor cells in order to survive, grow and metastasize. During their growth, tumor cells activate ECs by releasing various proteins, such as epidermal growth factor (EGF), fibroblast growth factor (FGF), interleukin-8 (IL-8), VEGF and others [98, 125]. Therefore, targeting only one of these factors is not sufficient to achieve an effective treatment and a combination of pharmacological agents targeting several factors is of great importance.

Indeed, hypoxia and insufficient vascularization in tumors lead to resistance to different types of damages given by different treatment regimens. As an example, VEGF mRNA levels were increased in hypoxic areas of glioblastoma, whereas vessels were found in close proximity of VEGF producing cells. The leaky vessels in turn lead to increased hypoxia and subsequently increased VEGF secretion and continuation of

the vicious cycle. One of the most clinically important features of a tumor is its capability to metastasize to other organs by taking advantage of increased vessel permeability. In this case, VEGF can promote the formation of the leaky vessels, which then can be infiltrated by tumor cells. However, there are other factors that can also induce new vessel formation in tumors [99, 126, 127].

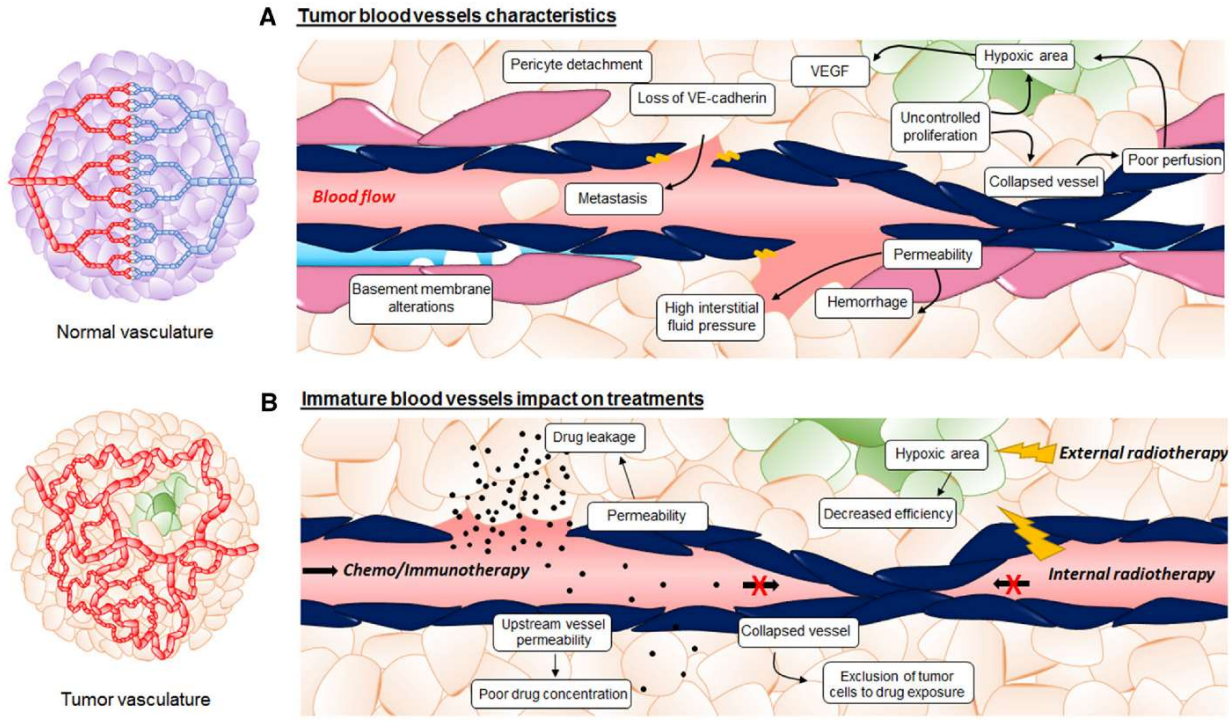

Figure 8. Tumor vasculature. A and B) immature vessels with decreased coverage by mural cells results in insufficient permeability leading to reduced perfusion and increased hypoxia. Some areas of the tumor are not sufficiently vascularized due to vessel collapse and therefore drugs cannot reach to the whole tumor. Figure adapted from [92].

1.5.3 Vascular endothelial factor (VEGF)

Among the angiogenic factors, VEGF is probably the most well-characterized. Through alternative splicing, six isoforms (121, 145, 165, 183, 189, and 206 amino acids) are generated from a single gene. Even though all isoforms demonstrate identical biological activities, VEGF165 is the most commonly expressed isoform [128]. VEGF is a dimeric glycoprotein [129], which is expressed by different tissues in low levels, is highly produced during angiogenesis as well as in majority of human tumors. VEGF binds to three known tyrosine kinase receptors: Flt-1 (VEGFR-1) [130], KDR/Flk-1

(VEGFR-2) [131], and Flt-4 (VEGFR-3) [132] and can be found on many cell types including vascular smooth muscle cells and monocytes/macrophages. It has been shown that VEGF mRNA is upregulated in many human cancers (breast, ovary, lung, kidney carcinomas, glioblastomas) and many cell lines secrete VEGF as demonstrated in *in vitro* studies [99]. The expression of VEGF and its receptor VEGFR-1 correlate with tumor vascularization, increased microvessel density (MVD) and poor prognosis for many patients [133, 134]. In addition, tumors can induce VEGF production in surrounding tissue, indicating the importance of VEGF for tumor angiogenesis. This has been demonstrated by inhibiting VEGF by an anti-VEGF antibody in sarcoma and glioblastoma xenografts in nude mice, which lead to reduced vessel density and inhibited tumor growth [135]. Similar results have been achieved inhibiting VEGF receptor in multiple tumor xenografts (lung, mammary, ovarian carcinomas) [99]. Additionally, VEGF can enhance permeability by relaxing junctions between ECs through reorganization of cadherin/catenin complexes. By inducing production of plasminogen activators (uPA, tPA), plasminogen activator inhibitor-1 (PAI-1) and interstitial collagenase, VEGF can stimulate remodeling of ECM components. VEGF stimulates EC proliferation and migration *in vitro* and *in vivo* [99], inhibits EC apoptosis and consequently acts as a survival factor [136]. The production of VEGF is also regulated by the availability of oxygen in the tissue [137]. Hypoxia stimulates VEGF production through the binding of HIF to the VEGF promoter, and increasing VEGF gene transcription and mRNA stability. While this mechanism guarantees that developing tissues and hypoxic areas become oxygenated, it also triggers pathological conditions (such as cancer) linked with angiogenesis [138].

VEGF receptors are characterized by seven extracellular immunoglobulin-like domains, a transmembrane domain, and a conserved intracellular tyrosine kinase domain [139]. VEGFR-1 is expressed in the endothelium as well as in healing skin wounds with high affinity for VEGF [99]. Despite its high affinity, VEGFR-1 does not mediate proliferation or migration directly. However, VEGFR-2, a tyrosine kinase with lower affinity for VEGF, facilitates chemotaxis, mitogenesis and shape alterations of ECs [140]. VEGFR-3 is mainly expressed by adult lymphatic endothelium and could be involved in lymphangiogenesis and binds VEGF-C and VEGF-D [141].

NRP1 is another receptor expressed on normal ECs, which binds VEGF165, enables its own binding to VEGFR-2, and augments chemotactic effects. VEGF was initially identified in tumor cell-conditioned medium as a protein that increased the

permeability of blood vessels. It can increase the permeability by improving the activity of clustered vesicles in ECs lining the vessels and enable transportation of metabolites between plasma membranes [99].

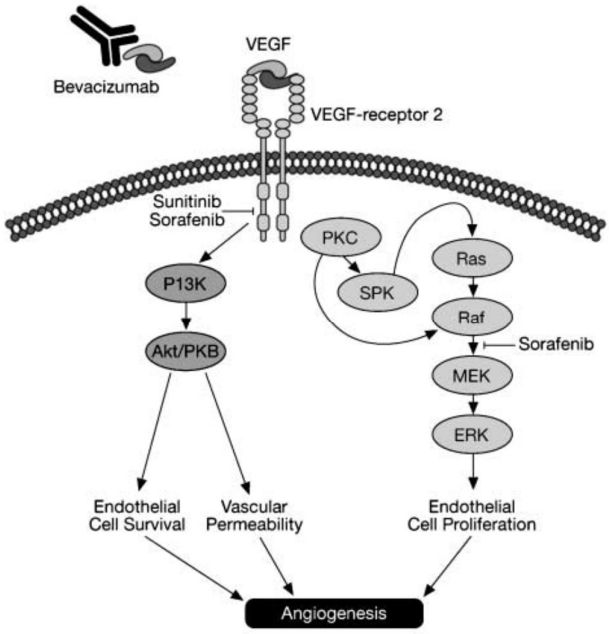

Figure 9. VEGF downstream signaling and its inhibitors. Binding of bevacizumab to VEGF inhibits its interaction with the receptors leading to inhibition of downstream signaling. Sunitinib and sorafenib, tyrosine kinase inhibitors, are capable of inhibiting different kinases of various downstream pathways after VEGF interaction. Figure adapted from [142].

1.5.4 Placental growth factor (PlGF)

Placental growth factor (PlGF) was first isolated from a term placenta cDNA library in 1991 as the second member of VEGF-family by Maria Graziella Persico in Napoli, Italy. They could identify a 149-amino-acid-long protein with 53% similarity to the platelet derived growth factor-like region of VEGF in the amino acid positions 39-132 [143]. In 1993, it was possible to locate the PlGF gene on chromosome 14q24, containing seven exons over 13.7 kb [144]. Due to alternative splicing, PlGF exists in four isoforms consisting of 131, 152, 203 and 224 amino acids [144, 145]. PlGF-2 and 4 have a heparin binding domain with 21 highly basic amino acids and bind to NRP1 and 2 expressed by multiple cells [144, 146-148]. PlGF is an N-glycosylated, homodimeric and secreted protein. Each monomer has six cysteine residues that form three intra-chain disulfide bonds, creating a cystine-knot motif. Two additional cysteine residues form two inter-

chain disulfide bonds stabilizing the homodimer. Residues Asp72 and Glu73, and glycosylated Asn84 in the N-terminal are important for receptor binding [143, 149-151]. PIGF is expressed during early embryogenesis and it is highly expressed in the placenta during the whole pregnancy regulating the growth and differentiation of trophoblasts [144]. Under normal conditions, PlGF expression is low in organs such as heart, skeletal muscle and lungs among others [152-154].

The receptors dimerize and phosphorylate upon ligand binding. PlGF can only bind to VEGFR-1 with higher affinity than VEGF-A [145, 155]. Compared to PlGF homodimers, PlGF-VEGF heterodimers are approximately up to 50 times less potent with regard to receptor binding and activation of downstream signaling pathways [156, 157]. Specifically, Ig-like domain 2 and 3 of the receptor are important for binding of PlGF, hence deletion of domain 3 leads to approximately 500-fold reduction in ligand binding [158]. By binding to VEGFR-1, PlGF can displace VEGF-A making it available for VEGFR-2, activate a cross-talk between the two receptors and leads to strong angiogenic signaling [110]. PlGF can also form a heterodimer with VEGF-A that is able to activate VEGFR-1 [155] or induce receptor heterodimerization [159].

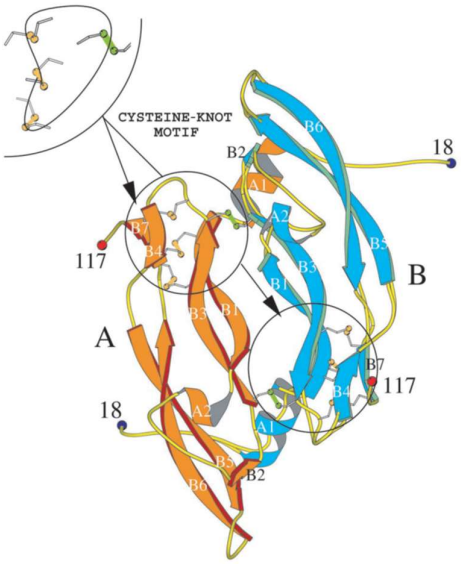

Figure 10. Model of PlGF-1 based on crystal structure. Representation of homodimer structure of PlGF-1 with disulfide bonds shown in yellow balls (intra-chain) and green balls (inter-chain) forming cysteine-knot motif. Each monomer is shown separately in different colors. Figure is adapted from [149].

In 1994-1996, the first PlGF expression experiments were performed demonstrating higher PlGF expression in renal cell carcinoma compared to surrounding normal tissue [160]. Tumor stage, metastasis and survival correlate to PlGF levels in tumors [161-163]. However, increased PlGF expression is not observed in all tumors [164].
In 1997, PlGF was shown to have angiogenic effects in a dose-dependent manner [155]. Even though PlGF is highly expressed in the placenta, its deletion did not affect embryonic development in mice [110]. Although PlGF deficient mice can develop normally, they are unable to adapt to pathological conditions such as inflammation or cancer. On the other hand, deletion of PlGF in adults impairs angiogenesis during pathological conditions such as tumor growth and ischemia [110, 165].
Carmeliet and colleagues have shown that PlGF can activate a crosstalk between VEGFR-1 and 2 and thereby boosts angiogenesis under pathological conditions [110, 165, 166]. They could demonstrate that PlGF phosphorylates a distinct tyrosine residue and activation of specific downstream pathways and subsequent gene expression. This in turn leads to increased VEGF-VEGFR-2 pathway and a separate angiogenic signaling [166]. Interestingly, heterodimers of PlGF-VEGF-A inhibit angiogenic functions of VEGF-A [159, 166].

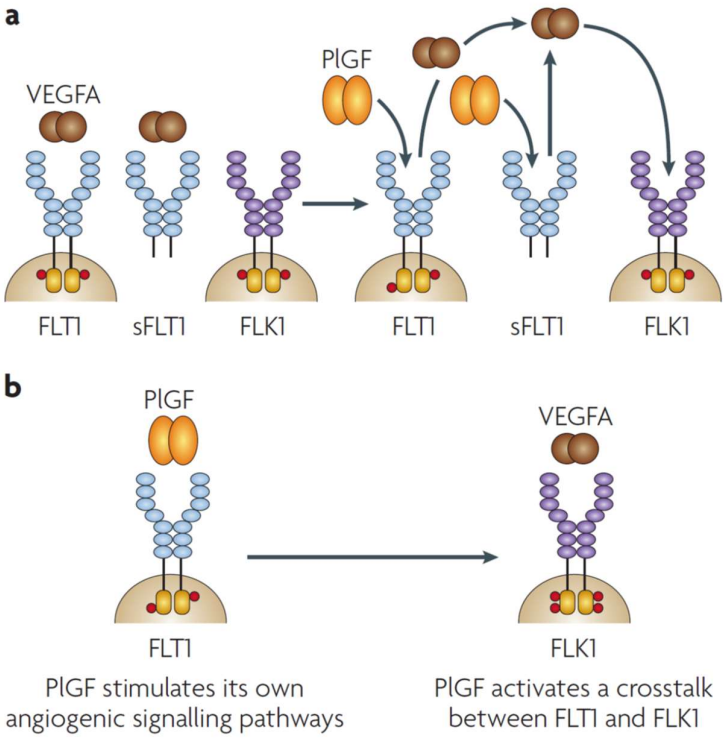

Figure 11. Molecular mechanisms of placental growth factor. A) PlGF binds only to VEGFR1 and its soluble version by displacing VEGF-A, making it available for VEGFR2. B) PlGF leads to phosphorylation of distinct tyrosine residues on VEGFR1 and stimulates its own angiogenic pathway. At the same time, this binding leads to crosstalk activation between the two receptor and a stronger angiogenic signaling. Figure adapted from [167].

PlGF can stimulate monocytes to secrete VEGF and increase the expression of IL-1β, IL-8 and VEGF [168]. It can recruit macrophages to tumors, wounds and ischemic areas [110, 165, 169].

These macrophages are able to inhibit dendritic cells, NK cells and CTLs leading to suppression of antitumor immune response [170]. Moreover, PlGF acts in a synergistic way with VEGF and PlGF deficiency correlates with diminished VEGFR-1, which has shown to delay wound healing [110]. Loss of PlGF abrogates the macrophage polarization and decreases vessel density even in presence of VEGF-A [160].

In several cancer types, increased PlGF expression correlates with tumor stage, metastasis and poor overall survival [167, 171]. Apart from the tumor cells, cells such as cancer associated fibroblasts (CAFs), ECs and tumor associated macrophages (TAMs) from the surrounding tissue can also express PlGF [110, 164, 165]. Consequently, PlGF generates an environment suitable for tumor cells to support their growth through different mechanisms, e.g. inducing vessel growth [172-174]. Interestingly, blockage of PlGF has shown to normalize vessels in different tumor models [174, 175]. On the other hand, a contradictory study shows tumor vessel normalization in PlGF overexpressing tumors [176]. According to several other studies, PlGF can also be an inhibitor of angiogenesis by creating PlGF/VEGF heterodimers when both of the genes are expressed [156, 157, 177]. The potency of the heterodimer was shown to be almost 50 times less than homodimers. Proliferation of human umbilical vein endothelial cells (HUVECs) in presence of homo- or heterodimers showed significantly decreased effect by PlGF/VEGF heterodimers only at high concentrations [109, 156, 177, 178].

Inhibition of VEGF and its receptors leads to increased PlGF levels in patients, probably leading to escape and resistance against these therapies [109, 171, 179, 180]. Blocking PlGF by RNA interference, neutralizing antibodies or gene silencing has shown decreased angiogenesis, tumor growth and metastasis in several mouse models [110, 164, 171, 174, 181-183]. Furthermore, a combined therapy with anti-VEGFR2 and

anti-PlGF antibodies was more effective than monotherapies. The effect of chemotherapy was improved upon PlGF inhibition with subsequent vessel normalization [164]. However, not all anti-PlGF antibodies have the same effect on tumors, which might partially depend on the expression of VEGFR-1 [175, 184, 185].

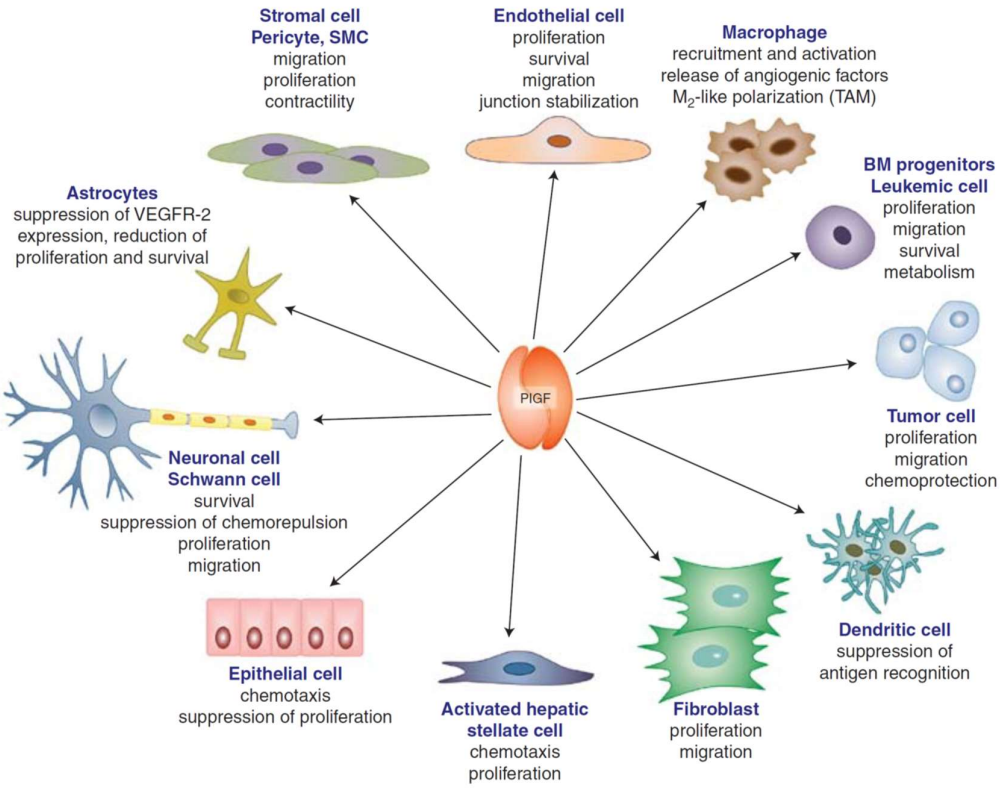

Figure 12. Pleiotropic effects of placental growth factor. As a cytokine, PlGF has various effect on survival, proliferation, migration, activation of vascular and nonvascular cells and metabolism. Figure adapted from [175].

1.5.6 Inhibitors of angiogenesis

1.5.6.1 Endogenous inhibitors

There are more than 40 endogenous inhibitors of angiogenesis which can be divided into 4 groups [186]:

Interferons. Interferons (INF-α, -β and γ) are secreted glycoproteins and have antiviral effect [187]. Moreover, IFN-α is able to inhibit tumor-induced migration of

ECs *in vitro* in a dose-dependent manner. In addition, IFN-α and IFN-β can downregulate bFGF mRNA and protein levels in several carcinoma cells [188].

Interleukins. Interleukins are secreted from leukocytes and have multiple functions, including activation and proliferation of lymphocytes [189] and stimulation of B cells to secrete IgE. Interleukins, such as IL-8, augments angiogenesis, whereas IL-4 has inhibitory effects [190] either by inhibiting tumor cell proliferation, inducing immune reaction or blocking EC migration [191].

Tissue inhibitors of metalloproteinases (TIMPs). It is known that the extracellular matrix plays a vital role in angiogenesis [99]. ECs use the components of the ECM, which have been modified by metalloproteinases to migrate and form vessels. This process is inhibited by TIMPs through their numerous effects on endothelial and tumor cells [192].

Proteolytic fragments. These fragments are cleaved from large proteins in the ECM (collagen, plasminogen, matrix metalloproteinases (MMPs), fibronectin) and possess antiangiogenic effects. For example, one of these fragments called angiostatin, a fragment of plasminogen, can inhibit the metastases by inhibiting EC growth. Fragment of type XVIII collagen called endostatin can inhibit the proliferation of EC both *in vitro* and *in vivo* [193].

Other antiangiogenic molecules. There are many factors, such as thrombospondin-1 (TSP-1), that can inhibit tumor angiogenesis, but further investigation is needed to characterize these factors. In one study, it was found that high level of TSP-1 was secreted only from non-tumorigenic cell line and could inhibit migration of ECs [99].

1.5.6.2 Pharmacological inhibitors of angiogenesis

There are several pharmacological inhibitors of angiogenesis that have been approved by the Food and Drug Administration (FDA). Anti-angiogenic drugs can lead to vascular normalization of tumor blood vessels [113] and improve the delivery of chemotherapeutic drugs and radiotherapy [194].

VEGF has been extensively studied since its involvement in angiogenesis was discovered in 1980s [195] and has been targeted to control tumor vessels. One of the most recognized agents against VEGF is a humanized anti-VEGF antibody

Bevacizumab, which blocks secreted VEGF leading to inhibition of new vessel formation and tumor growth [196, 197]. Its effect in combined treatment modalities has been investigated in detail. Bevacizumab combined with chemotherapy has shown improved survival in gastric cancer, NSCLC and metastatic breast cancer [63, 198, 199]. However, several side effects (bleeding, hypertension and thromboembolism) of the drug were observed when combined with other treatment regimens in different cancers, even though combination with carboplatin and paclitaxel showed improved overall response [200-203].

In addition to Bevacizumab, agents targeting VEGFRs (e.g. Sunitinib, Sorafenib) have also been developed for treatment of cancer treatment [204]. However, the effect of these agents depend on tumor type, as prostate and pancreatic cancers do not show sensitivity to these treatments [205]. Sunitinib (SU11248) is a tyrosine kinase inhibitor (TKI) used for treatment of kidney cancer renal cell carcinoma (RCC) and imatinib-resistant gastrointestinal tumors. It has also shown favorable results in many cancers [98]. Sorafenib (Nexavar) is also a TKI, approved for hepatocellular carcinoma [206], advanced renal cell carcinoma [207], and thyroid carcinoma [208] treatment.

Increased VEGF secretion from tumor cells leads to immature blood vessels. Treatment with VEGF inhibitors balances angiogenic processes leading to mature vessel network and improved perfusion [209]. Therefore, improvement in the combination therapy with VEGF inhibitors and radio/chemotherapy might be due to vessel normalization [113]. However, wrong dosage or longer treatments with these inhibitors causes regression of the vascular network increasing the risks for metastatic disease [210].

The inhibitors used to treat tumor angiogenesis might also hamper normal angiogenic processes, such as blood pressure, reproduction, wound healing and embryonic development [211, 212]. For example, one of the prevalent side effects in patients treated with inhibitors of VEGF signaling is increased blood pressure. Briefly, VEGF is involved in upregulating endothelial nitric oxide synthase (eNOS) and prostacyclin (PGI2) in ECs by activating MAPK and PI3K pathways, releasing nitric oxide (NO) and resulting in vessel dilation [105, 213, 214]. Consequently, inhibition of VEGF leads to decreased NO production, which then induces vessel contraction and elevate blood pressure [215]. Inhibition of VEGF can also lead to delays or complications in wound healing [216].

Apart from their toxicity, a prolonged treatment with VEGF inhibitors show increased VEGF-A, -B, -C, PlGF expression, and phosphorylation of their receptors [217]. Nevertheless, treatment with anti-angiogenic agents results in a normalized vasculature that might allow for better delivery of drugs [98].

Radiation on its own induces vessel damage and leads to substantial changes in tumor vasculature, blood flow and oxygenation [218]. These changes can be influenced by treatment dose, radiation quality and fractionation among others, and are responsible for the variances in treatment response. However, using anti-angiogenic drugs results in vessel normalization, reduced hypoxia, improved oxygen levels and subsequently more effective treatment response when combined with RT [219].

Generic Name	FDA-Approved Indication
Bevacizumab	Colorectal, non-small-cell lung, and glioblastoma multiforme
Thalidomide	Myeloma
Lenalidomide	Myeloma (myelodysplastic syndrome (MDS))
Sorafenib	Renal cell and hepatocellular carcinoma
Sunitinib	Renal cell and gastrointestinal carcinoma
Temsirolimus	Renal cell carcinoma
Axitinib	Renal cell carcinoma
Pazopanib	Renal cell carcinoma, kidney cancer, and advanced soft tissue sarcoma
Cabozantinib	Thyroid cancer
Everolimus	Kidney cancer, advanced breast cancer, pancreatic neuroendocrine tumors (PNETs), and subependymal giant cell astrocytoma
Ramucirumab	Stomach cancer and gastroesophageal junction adenocarcinoma
Regorafenib	Colorectal cancer and gastrointestinal stromal tumor
Vandetanib	Thyroid cancer
Ziv-aflibercept	Colorectal cancer

Figure 13. A list of FDA-approved anti-angiogenic drugs that can be used in combination with other therapies. Figure adapted from [98].

1.5.6.3 Alternative targets

Despite the advantages with VEGF signaling inhibitors, several serious side effects, such as hemorrhage and venous thromboembolism can be observed [220]. Another severe side effect includes tumor metastasis due to degeneration of normalized tumor blood vessels [92]. Lastly, treatment with VEGF inhibitors result in temporary effects which can be overcome by resistance mechanisms in tumor cells [179]. Therefore, there is an urgent need to find other targets.

An important modulator of tumor angiogenesis is PDGF-B and its receptors PDGFRa/b, closely involved in blood vessel maturation. ECs secrete PDGF-B, which

binds to its receptors on pericytes and recruits them to the blood vessels. Combined inhibition of PDGFRb with SU6668 and VEGFR with SU5416 demonstrated reduced tumor size and improved the effect of radiotherapy [92].

Other key players in blood vessels formation are the angiopoietins and Tie2 receptors. Angiopoietins bind to Tie2 on ECs leading to their survival, migration and vessel formation through receptor phosphorylation and subsequent signaling pathway activation [221-223]. Agents targeting the angiopoietin/Tie2 pathways are being investigated, where an antibody (MEDI3617) blocking Ang2 shows decrease in lung metastasis and tumor associated angiogenesis in several tumor models [224]. Moreover, combination therapy with VEGFR and Ang2 inhibitors demonstrated improved survival in glioblastoma models [225].

VE-cadherin is an important regulator of cell-cell junctions between ECs and blood vessel permeability. Sac-1004 is a molecule upregulating VE-cadherin through activation of the cAMP/Rac/Cortactin pathway and thereby improves the perfusion, decreases metastasis and improves chemotherapy treatment in mouse models [92].

2 Aims of Study

Radiation therapy is an essential treatment method for cancer management. It allows precise radiation dose distribution to the tumor while sparing the normal tissue as much as possible. In addition to DNA damage and cancer cell killing, radiation therapy also leads to secretion of several factors from cancer cells as part of a stress response. Our laboratory has been interested in identifying these factors and unraveling ionizing radiation induced treatment resistance. One of the factors identified in our large-scale secretome analysis was placental growth factor (PlGF), which is known to be involved in pathological angiogenesis and is the focus of this PhD thesis. The mechanisms behind this IR-induced signal transduction cascade are not fully understood. Therefore, in the current PhD project, we aim to investigate these signaling pathways and resulting cellular responses more in detail in order to understand the role of PlGF as a rescue mechanism to irradiation.

Based on our results and knowledge, the first part of this project concentrated on the characterization of the PlGF response and its regulation in irradiated tumor cells. We investigate the expression and secretion of PlGF in response to increasing doses of IR in several tumor cell lines with differential genetic backgrounds. In order to determine the relevance of signal transduction for IR-induced PlGF-expression, parallel experiments in presence of respective small molecular inhibitors are performed. Likewise, the expression and secretion of PlGF alone and in combination with irradiation under hypoxia is investigated in order to show a differential regulation of PlGF.

The ionizing radiation induced role of PlGF is not fully understood and so far no data is available *in vitro* or *in vivo*. Therefore, in the second part of the project, we explore the role of PlGF as rescue mechanism in response to IR by investigating its paracrine effects *in vitro* using PlGF wildtype or knockout cells. We also investigate the role and relevance of PlGF on the tumor vasculature and on the tumor sensitivity to ionizing radiation *in vivo*.

3 Results

3.1 The Microtubule Targeting Agent BAL101553 Provides Superior AntiTumor Activity in Combination with Bevacizumab and has Differential Effects on Tumor Vascularization with i.v. and Oral Dosing

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Status of the manuscript: Ready for submission

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- Data acquisition, analysis and interpretation of experiments
- Figure 1: contributed regarding Western blot and ELISA in BAL 27862-treated A549, A549EpoB40 and SW480 cells
- Revision and proofreading of the manuscript

The Microtubule Targeting Agent BAL101553 Provides Superior Anti-Tumor Activity in Combination with Bevacizumab and has Differential Effects on Tumor Vascularization with i.v. and Oral Dosing

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Running title: BAL101553 in combination with bevacizumab

Keywords: BAL101553, Microtubule Targeting Agent, Combined Treatment Modality, Bevacizumab

Total number of figures: 7 Figures

Supplementary material: 5 Suppl. Figures

Abstract

Background: BAL101553 is a highly soluble prodrug of BAL27862, a novel, small molecule, microtubule-depolymerizer inducing tumor cell death through the 'spindle assembly checkpoint'. BAL101553 has anti-cancer activity in diverse tumor models refractory to standard therapies, and is currently undergoing Phase 1/2a evaluation in advanced cancer patients with IV (weekly) and oral (daily) dosing schedules. Here, the vascular disruption activity of BAL101553 was evaluated in the context of administration route and implications for combinations with VEGF-targeted therapies.

Methods: Drug combinations of clinically relevant BAL101553 (MTD i.v. or Oral daily) and antiangiogenic agents (Bevacizumab or Everolimus) were investigated in genetically defined MTA-resistant lung (A549EpoB40) and colon adenocarcinoma (SW480) tumor xenografts in nude mice. We employed endothelial tube formation, and secreted biomarker screening to determine the functional relevance of combined treatment. Tumors were analyzed by IHC for proliferation (Ki67), tumor hypoxia (Pimonidazole, Carbonic anhydrase-IX) and vascularization (CD31, lectin perfusion).

Results: Sub-cytotoxic BAL27862 concentrations exerted a broad-spectrum inhibitory activity on HIF1α protein levels and HIF1α-controlled secretome *in vitro* and *in vivo*. Interestingly, secretome derived from BAL27862-treated tumor cells diminished endothelial tube formation, which could be partially rescued by exogenous VEGF, indicating direct interference of the drug on hypoxic adaptation and endothelial tube formation. SW480- or A549EpoB40-derived tumor xenografts received once (i.v. 25 mg/kg; high Cmax regimen) or daily (oral 25 mg/kg; low Cmax, high AUC) BAL101553 regimens which elicited partial anti-proliferative and antivascular effects. Based on these data, combination of the anti-VEGF antibody bevacizumab with BAL101553 was assessed in the paclitaxel refractory SW480 and the epothilone resistant A549EpoB40 tumor models, using MTD BAL101553 dosing (i.v. weekly & oral daily). All monotherapies induced an anti-tumor response. However, bevacizumab combined with IV or oral BAL101553 was superior to single agents with the IV combination associated with the least functional tumor vasculature.

Conclusions: BAL101553 targets tumor cell proliferation, tumor hypoxic adaptation and vascularization. The latter is Cmax-driven and can be attenuated by daily oral dosing, providing an alternative pharmacodynamic endpoint with implications for clinical treatment strategies.

IV and orally administered BAL101553 both elicit superior anti-tumor responses in combination with anti-VEGF therapy.

Introduction

Microtubule targeting agents (MTA) are a mainstay in cancer treatment with a curative intent or aim to palliate symptoms and to prolong life. The clinical utility of MTAs is often limited by MTA-related toxicities and resistance to treatment; the latter is associated with β-tubulinrelated mutations, overexpression of P-glycoprotein, and altered cellular β-tubulin isotype composition [1]. Thus, discovery of novel MTAs alone and in combination with other clinically relevant treatment modalities are of high demand to overcome intrinsic or acquired resistance mechanisms.

Therapeutic response and clinical outcome are not solely determined by genetic alterations and acquisition of mutations in the neoplastic cells, but also by the fitness advantage conferred by the given tumor microenvironment [2]. Tumor cells are the primary targets for classic chemotherapeutic treatment. Besides their direct tumor-cell directed cytotoxicity, MTAs are also antiangiogenic and show antivascular activity. We and others previously demonstrated that MTAs downregulate the HIF-transcriptome and interfere with the secretion of multiple tumor cell-derived factors, e.g. vascular endothelial growth factor VEGF and stress-induced lysyl oxidase LOX [3, 4]. Thus, the tumor growth-inhibitory effect of MTAs in MTA-sensitive tumors might at least in part be due to their indirect, tumor cell-mediated effect on the tumor microenvironment [5].

BAL101553 is a highly soluble prodrug of BAL27862, a novel, small molecule, microtubuledepolymerizer inducing tumor cell death through the 'spindle assembly checkpoint'. BAL27862 binds the colchicine site of tubulin with distinct effects on microtubule organization, resulting in tumor cell death [6]. BAL101553 has anti-cancer activity in diverse tumor models refractory to standard therapies, and is currently undergoing Phase 1/2a evaluation in advanced cancer patients with i.v. (weekly) and oral (daily) dosing schedules [7, 8]. Here, we study the anti-tumor activity and vascular disruption property of BAL101553 in the context of administration route and implications for combinations with VEGF-targeted therapies.

Materials and Methods

Cell culture and Compounds

SW480 human colon adenocarcinoma, A549 and A549EpoB40 human lung cancer cells were cultured in RPMI1640 media supplemented with 10 % (v/v) fetal calf serum, 1 % (v/v) penicillin-streptomycin and 1 % (v/v) L-glutamine at 37°C in 5 % CO2. A549EpoB40 cells

were maintained in the presence of 10 nM epothilone B. BAL27862 and BAL101553 were provided by Basilea Pharmaceutica International Ltd (Basel, Switzerland). BAL27862 (10 mM stock) is used for in vitro experiments and is the active moiety of the lysine prodrug BAL101553.

Western blotting

For western blot analysis, A549, A549EpoB40 and SW480 cells were preincubated with BAL27862 (24 h) or RAD001 (1 h) followed by additional incubation for 6 hours under normoxic or hypoxic conditions. BAL27862- and RAD001 (Everolimus)-treated tumor cell lysates were tested against HIF1α subunit (Santa Cruz Inc., #sc-10790) and anti-β-actin antibody (Sigma Aldrich, #A5441) by western blotting. Antibody detection was achieved by enhanced chemoluminescence (Amersham, Piscataway, NJ) according to the protocol of the manufacturer.

Bioplex Biomarker Assay and ELISA

Tumor cells were preincubated with BAL27862 (24 h) followed by additional incubation for 24 h under normoxic or hypoxic (0.2%) conditions. Customized Bioplex Biomarker Cancer Panel assay (17 biomarkers) was performed with undiluted conditioned medium samples according to the manufacturer protocol (Bio-Rad). The concentration of VEGF levels in the filtered, conditioned media of A549EpoB40 cells was determined using the Quantikine® Human VEGF Immunoassay kit (R&D Systems GmbH).

Endothelial tube formation assay

The endothelial tube formation assay was performed on the µ-Slide Angiogenesis platform according to the protocol provided by the manufacturer (ibidi, #81506). 10 µL of Matrigel (BD Basement Membrane Matrix, #354234) was loaded in each well of the 15-well µ-Slide and HUVECs were plated at the appropriate cell density (10,000 cells/well). Capillary-like tube formation was observed after 16 h using a microscope (Axiovert 40 CFL, Zeiss with Axiocam) and quantified using Angiogenesis Analyzer from ImageJ (NIH). Original images (5 images per treatment group) were transformed to binary images and further skeletonized to compute parameters such as number of segments (Nb segments) and meshes (Nb mesh) using Angiogenesis Analyzer plugin of ImageJ (NIH) software.

Tumor xenografts

Tumor xenografts derived from A549EpoB40 and SW480 cells in athymic nude mice were generated as described in [9] and allowed to expand to a volume of 200 mm3 (±10%). BAL101553 (dissolved in buffered saline) was applied as either a bolus (i.v. 21.3 mg/kg, once a week over three weeks) or daily p.o. (oral 15 mg/kg, 5 days/week over three weeks). Bevacizumab (Avastin, Roche, concentrated infusion solution) was diluted with saline and injected i.p. at 5 mg/kg twice on day 1 and day 4 (in both SW480 and A549EpoB40 xenografts). Control mice received a corresponding placebo-treatment (buffered saline). This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Swiss Cantonal Veterinary Authorities.

Immunohistology

Immunohistological endpoints were analyzed on formalin fixed and paraffin embedded blocks for Ki-67 (prediluted; Ventana-Roche, 790-4286), CD31 (1:10; Dako, M0823), IL8 (1:20; R&D Systems, MAB330), Pimonidazole (1:100; Hypoxyprobe, HP1-1000), and Carbonic anhydrase (1:6000; Abcam, ab15086) using a Discovery Immunohistochemistry Staining System (Ventana Medical Systems). Ki-67–positive tumor cells and amount of vessels (CD31) were counted in at least four randomly chosen visual fields (magnification, 400X) in each xenograft (n=4 for each group). Mice were injected with FITC_Agglutinin (i.v. 100 µg/mouse) 1 hour before tumor harvesting and % of functional perfused vessels were calculated as ratio of Agglutinin+CD31+ vessels to total CD31+ vessels. Whole tumor sections were quantified for specific H&E, Pimonidazole or CA-IX staining intensity employing a 2000 µm manual counting grid (n=4 for each treatment group).

Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 5). If not indicated otherwise, in vitro data were analyzed using the unpaired Student t test and data represent at least three independently performed assays. In vivo treatment response was evaluated by two different statistical metrics. Areas under the tumor volume curve (AUC) were analyzed by oneway ANOVA using the Tukey Test for pair-wise comparisons. Additionally, tumor growth delays were also calculated by Kaplan Meier analysis with Log-rank (Mantel-Cox) test for pairwise comparisons. Immunohistochemical data were analyzed by one-way ANOVA on replicate tumor samples (n=4) and pair-wise analysis was performed using the Tukey Test. For all experiments, *P<0.05, **P<0.01, ***P<0.001.

Results

BAL27862 decreases HIF1α levels and subsequent downstream VEGF-expression and secretion

Our previous work demonstrated that the tumor growth-inhibitory effect of MTAs in MTAsensitive tumors might at least in part be due to downregulation of the HIF-transcriptome thereby contributing to an indirect, tumor cell-mediated effect on the tumor microenvironment [4, 5] To probe for its HIF-repressive capacity, the novel microtubule-destabilizing agent BAL27862 was tested for its HIF-interfering capacity in MTA-resistant colon and NSCLC tumor cell lines.

Parental A549, PgP-overexpressing paclitaxel-resistant SW480 and epothilone resistant (βtubulin mutated) A549EpoB40 cells were preincubated with BAL27862 (24 h) followed by 6 h incubation under normoxic or hypoxic (0.2% oxygen) conditions (IC50s: 28 nM, 9 nM & 22 nM, respectively; [10]). Western blot analysis of whole cell lysates revealed potent downregulation of HIF1 levels after BAL27862 treatment in all the three cell lines (Figure 1A). Additionally, VEGF secretion was significantly upregulated under hypoxia, but not in cells pretreated with subtoxic concentrations of BAL27862 (10 nM, Figure 1B). These results indicate that an intact microtubular network is required for HIF1α stabilization under hypoxia and subsequent VEGF-secretion. Cellular preincubation with the microtubule-destabilizing agent BAL27862 is sufficient to interfere with HIF1α stabilization, presumably through deregulated HIF1α -expression.

BAL27862 exerts a broad-spectrum inhibitory activity on HIF1α-controlled secretome in vitro and in vivo

We previously identified that MTAs indirectly affect the tumor microenvironment via regulation of tumor cell-derived secretory factors [5]. Secreted factors are key mediators of cellcell communications and their altered levels enable tumor progression and therapy resistance [3, 11, 12]. Therefore, Bioplex-based exploratory analysis was performed to assess secretion status of 17 key cancer biomarkers in response to subtoxic levels of BAL27862 (10 nM) in SW480 and A549EpoB40 tumor cells under both normoxic and hypoxic (0.2%) conditions. Interestingly, BAL27862 exerted a broad-spectrum inhibitory activity on the HIF1α-controlled secretome resulting in reduced secreted levels of HIF1α-downstream factors such as VEGF, IL8, OPN (Osteopontin), SCF (Stem cell factor), and PAI-1 (Plasminogen activator inhibitor1) (Figure 2A and 2B). Similar HIF1α-downstream inhibitory activity could

also be determined in the SW480 cells with increased concentration of BAL27862 (20nM, Supplementary Figure 1A).

To confirm our findings in vivo, we developed tumor xenografts derived from taxane-resistant SW480 and patupilone-resistant A549EpoB40 tumor cells and treated mice with either a bolus (i.v. 21.3 mg/kg, day 1) or a daily p.o. (oral 15 mg/kg, day 1 - 5) regimen of BAL101553. Tumors and blood sera were harvested on day 7 after treatment. Ex vivo analysis of blood serum from BAL101553-treated SW480- and A549EpoB40-derived tumor xenografts revealed significantly reduced levels of IL8 in comparison to blood serum levels from vehicle-treated mice (Figure 2C and 2D). Unfortunately, VEGF concentrations were below a minimal technically detectable concentration.

Additionally, we also observed reduced immunohistochemical staining of IL8 in tumor sections derived from BAL101553-treated xenografts in comparison to vehicle-treated tumors (Figure 2E and 2F). Of note, TNFα (Tumor necrosis factor alpha), a hypoxia independent marker did not change in response to this novel MTA indicating specific HIF1α-mediated inhibitory activity of BAL27862 and BAL101553 in vitro and in vivo, respectively, (Supplementary Figure 1B and 1C).

These results demonstrate that BAL27862 exerts a broad-spectrum inhibitory activity on the HIF1α-controlled secretome across a panel of MTA-resistant cell lines and tumor xenografts.

The secretome of BAL27862-treated tumor cells diminishes endothelial tube formation, which can be partially rescued by exogenous VEGF

The role of a deregulated HIF1α-downstream secretome was studied in vitro by investigating tube formation in HUVECs. The secretome (conditioned medium) derived from BAL27862treated A549 cells diminished capillary-tube formation and vascular organization of HUVEC cells in a paracrine manner mimicking a phenotype similar to direct treatment of HUVECs with the anti-VEGF targeting antibody bevacizumab (Figure 3A). The inhibition of endothelial tube formation could be partially rescued by exogenous addition of VEGF suggesting that the VEGF signaling pathway is partially required for tube formation in this assay system (Figure 3B and 3C).

Modest proliferation and mean vessel density decrease in SW480 and A549EpoB40 tumors after short-term i.v. and oral BAL101553 treatment

Our own pharmacokinetic and pharmacodynamic experiments performed in preclinical tumor models with BAL101553 alone revealed two near-equipotent treatment regimens of BAL101553 [i.v. once (21.3 mg/kg) and oral daily (5 x 15 mg/kg)], with increased tolerability with the oral route [7, 10]. Equivalent and statistically significant reductions in the proliferation indices (Ki-67) and mean vessel density (CD31) were observed in both the treatment groups after BAL101553 treatment in both SW480- and A549EpoB40-derived tumor xenografts (Figure 4A and 4B). However, determination of treatment-related body weight changes revealed a considerable BAL101553-dependent transient weight loss over 48 h after i.v. bolus but not during the oral daily regimen indicating lesser toxicity and better tolerability with the oral regimen in these tumor models (Suppl. Figure 2A and 2B).

Although single BAL101553 treatment in these tumor models display clear anti-tumor and antivascular activity, we speculated that combining BAL101553 with an antiangiogenic agent could drastically improve treatment response and outcome while effectively managing any additive or synergistic toxicities.

Combination of i.v. or oral BAL101553 with bevacizumab in MTA-resistant tumor xenografts

To assess the anti-tumor efficacy of BAL101553 with its indirect anti-vascular activity in combination with direct anti-angiogenic targeting, the combination of the anti-VEGF antibody bevacizumab with BAL101553 was assessed in the paclitaxel refractory SW480 and the epothilone resistant (β-tubulin mutated) A549EpoB40 tumor model, using equipotent MTD BAL101553 dosing (i.v. weekly & oral daily). In the taxane-resistant SW480 tumor model, all monotherapies induced an anti-tumor response (ΔT/Cs bevacizumab: 35%; i.v. BAL101553: 22%; oral BAL101553: 53%). However, bevacizumab combined with i.v. or oral BAL101553 was superior to either of the single agents (ΔT/C: 6% and 17%, resp.) (Figure 5A). Furthermore, the absolute growth delay to triple the initial tumor volume (200 mm^3) was most enhanced in response to the combined treatment modality when compared with the absolute tumor growth delay in response to i.v. or oral BAL101553 or bevacizumab alone ($P < 0.01$ for i.v. or oral BAL101553 plus bevacizumab versus vehicle and each monotherapy), respectively, suggesting a positive interaction between BAL101553 and bevacizumab (Suppl. Figure 3A).

While SW480 cells overexpress the multidrug resistance (MDR)–related efflux pump Pglycoprotein, thereby strongly reducing the potency of P-glycoprotein substrates such as taxanes, many tumors carry a specific mutation in the MTA binding region of β-tubulin, leading to epothilone and taxane resistance. We probed the strategy to combine BAL101553 with bevacizumab also in the defined β-tubulin mutated carcinoma tumor model (A549EpoB40). In the A549EpoB40 tumor model, all monotherapies induced an anti-tumor response (ΔT/Cs bevacizumab: 57%; i.v. BAL101553: 49%; oral BAL101553: 57%). However, bevacizumab combined with i.v. or oral BAL101553 was superior to either of the single agents (ΔT/C: 9% and 22%, resp.) (Figure 5B).

Downregulation of the HIF-transcriptome with subsequent reduction of tumor-derived VEGF expression and secretion could also be executed by the mTOR inhibitor everolimus instead of bevacizumab in MTA-resistant tumors. Surprisingly, we did not observe any additive tumor growth delay in response to the combination of BAL101553 (i.v. weekly or oral daily) and Everolimus (Figure 5C), even though treatment with everolimus, a PgP-substrate, led to significant downregulation of phosphorylated P70-S6Kinase in these SW480 tumors (Suppl. Figure 3B).

Determination of treatment-related body weight changes revealed a minor BAL101553dependent transient weight loss over 48 hours after i.v. bolus but not during the p.o. daily regimen (Suppl. Figure 3C and 3D). Analysis of tissue morphology and integrity of skin adjacent to the tumor did not reveal any normal tissue toxicities in response to the combined treatment modality (data not shown).

Induction of tumor necrosis, reduced proliferation and enhanced anti-vascular effects with BAL101553/bevacizumab combinations in MTA-resistant tumor xenografts

To determine the anti-vascular effect of the different treatment modalities in situ, microvessel density (CD31) and perfusion (agglutinin perfusion), necrosis (H&E), proliferation (Ki67) and tumor hypoxia (Pimonidazole and carbonic anhydrase) were determined in tumor sections (Figures 6, 7). Interestingly, tumor necrosis was dramatically increased in both combination groups (BAL101553 administered i.v. weekly or oral daily with bevacizumab) as compared to the single agents in both tumor models (SW480, Figure 6, 7A; A549EpoB40 Figure 7D and Supplementary Figure 4A).

Moreover, reductions in proliferation and mean vessel density within viable areas of the tumor were also observed in the combination groups (SW480, Figure 6, 7B; A549EpoB40 Figure 7E

and Supplementary Figure 4). Strikingly, i.v. BAL101553 in combination with bevacizumab was associated with the least functional tumor vasculature (based also on vascular perfusion experiments) whereas the daily-oral BAL101553 regimen had the least anti-vascular activity, despite imparting similar anti-tumor activity in combination (Figure 7C and 7F).

IL8 (CXCL8) as a novel predictive circulating biomarker for efficacy of combined BAL101553 and Bevacizumab regimen

Secreted serum components can be associated with treatment efficacy and could potentially participate in the generation of de novo resistance mechanisms. We performed analysis of blood serum derived from SW480 and A549EpoB40 tumor xenografts. Tumors and blood sera were harvested on day 7 after treatment with either bolus BAL101553 (i.v. 21.3 mg/kg, day 1) or daily BAL101553 (oral 15 mg/kg, day 1 - 5) and Bevacizumab (i.p. 5 mg/kg, day 1 and 4). Ex vivo analysis of blood serum from BAL101553-treated SW480- and A549EpoB40-derived tumor xenografts revealed significantly reduced levels of IL8 in comparison to blood serum levels from vehicle-treated mice. Importantly, combined treatment with BAL101553 (i.v. or oral daily) and bevacizumab also led to significantly reduced serum levels of IL8 in the blood serum of mice in comparison to bevacizumab or vehicle-treated tumors (Figure 7G and 7I). Additionally, we also observed reduced immunohistochemical staining of IL8 in tumor sections derived from combined BAL101553 and bevacizumab-treated xenografts in comparison to monotherapy or vehicle-treated tumors (Figure 7H and Supplementary Figure 5). These results suggest that post-treatment IL8 serum levels may be associated with the efficacy of BAL101553 and bevacizumab-containing regimen.

Discussion

Therapy resistance invariably limits the clinical efficacy of microtubule targeting agents against advanced metastatic cancers. Influence of chemotherapeutic resistance, whether intrinsic or acquired is drastic as it causes treatment failure in over 90% of patients with metastatic cancer and can hamper the effectiveness of the therapy even in the adjuvant setting [13]. Antiangiogenic tumor therapies, especially targeting the VEGF pathway, have shown statistically significant and clinically meaningful improvement in survival among metastatic advanced non-small cell lung and colorectal cancer patients [14, 15].

We previously identified that bevacizumab in combination with epothilone or taxane can overcome MTA-resistance in otherwise MTA-refractory tumor models [5]. We have here

demonstrated the potency of the combined treatment modality of novel the MTA BAL101553 with bevacizumab, that is active both in taxane-resistant and β-tubulin-mutation-linked epothilone-resistant tumor cells [7, 8].

BAL101553 is a small molecule tumor checkpoint controller, currently in clinical phase 1/2a evaluation in patients with advanced solid tumors or glioblastoma (brain cancer). BAL101553 binds to tubulin at a site not targeted by any approved microtubule-targeting agent and demonstrates efficacy against diverse treatment-resistant cancer models, including tumors refractory to conventional approved therapeutics and radiotherapy [6-8].

Due to its intravenous and oral bioavailability, different dosing regimens for BAL101553 are being assessed (Trial IDs: NCT01397929, NCT02895360, NCT02490800). While the i.v. bolus regimen might be more directly cytotoxic and antivascular, the daily-oral route application might induce a pseudo-metronomic effect. Of note, currently approved MTAs cannot be administered clinically with a daily oral schedule.

Serum circulating cytokines and angiogenesis-related secreted factors are hypothesized to be valid biomarkers for the angiogenic and hypoxic status of the tumor microenvironment and may offer prognostic and predictive information beyond conventional clinico-pathological indicators [16]. Based on an explorative secretome screening, we identified that post-treatment IL8 serum levels may be associated with the efficacy of BAL101553 and bevacizumabcontaining regimens.

Overall, our results clearly demonstrate that BAL101553 targets tumor cell proliferation, tumor hypoxic adaptation and vascularization. IV and oral administration of BAL101553 provides an alternative pharmacodynamic endpoint with implications for clinical combined treatment strategies with anti-VEGF therapies.

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Figure Legends

Figure 1. BAL27862 inhibits HIF1α levels and subsequent downstream VEGF-expression and secretion. (**A**) BAL27862- and RAD001-treated A549, A549EpoB40 and SW480 cell lysates were tested with an anti-HIF1α subunit antibody by western blotting. Cells were preincubated with BAL27862 (24 h) or RAD001 (1 h) followed by additional incubation for 6 hours under normoxic or hypoxic conditions. (**B**) Regulation of VEGF secretion by BAL27862. Tumor cells were treated with BAL27862 (10 nM, 24 h) and cultured under normoxic and hypoxic (0.2%). Supernatants were collected 24 hours after starting hypoxic conditions and analyzed for VEGF protein levels by ELISA. Unpaired Student t test, data are represented as mean ± SD. *, $P < 0.05$.

Figure 2. BAL27862 exerts a broad spectrum inhibitory activity on HIF1α-controlled secretome. Cancer biomarker screening was performed with Bioplex multiplex technology against conditioned media (CM) derived from untreated and BAL27862-treated SW480 colon carcinoma (**A**) and A549EpoB40 lung adenocarcinoma (**B**) cells. Cells were preincubated with BAL27862 (24 h) followed by additional incubation for 24 hours under normoxic or hypoxic (0.2%) conditions. (**C-D**) Serum levels of IL8 in murine blood derived from i.v. BAL101553 (21.3 mg/kg, day 1), oral BAL101553 (15 mg/kg, day 1 - 5) or vehicle-treated SW480- and A549EpoB40-derived tumor xenografts. Tumors and blood sera were collected 7 days after treatment start. (**E-F**) Representative images and quantification of immunohistochemical staining intensity for IL8 in tumor sections derived from SW480- and A549EpoB40 tumor xenografts (n=4). Viable tumor sections were evaluated for specific IL8-staining intensity. Scale bar 100 μm. One-way ANOVA, data are represented as mean ± standard deviation. *$P<0.05$.

Figure 3. The secretome of BAL27862-treated tumor cells diminishes endothelial tube formation, which can be partially rescued by exogenous VEGF. A549 cells were pretreated with different indicated concentrations of BAL27862 (0 nM, 5 nM or 20 nM) for 24 hours and then replated in fresh EGM media for 16 hours for conditioning. Naïve HUVEC cells were treated with either VEGF (100 ng/ml), bevacizumab (50 μg/ml) or with conditioned media (CM) collected as explained above. The formation of capillary-like networks was acquired after 12 hours by inverted microscopy (5 replicates per group) and analyzed using the Angiogenesis Analyzer ImageJ toolkit. (**A**) Number of meshes and segments of the HUVEC-tubes after treatment as described above. (**B-C**) Representative images and quantification of the HUVEC tube formation in response to conditioned media (CM) derived from untreated- and BAL27862-treated (20 nM) A549 cells, with and without exogenous addition of VEGF (100 ng/ml). Unpaired Student t test, data are represented as mean ± SD. *, $P < 0.05$.

Figure 4. Partial proliferation and mean vessel density decrease in SW480 and A549EpoB40 tumors after short-term BAL101553 treatment. (**A**) SW480- or (**B**) A549EpoB40-derived tumor xenografts (n = 4 per group) were treated with either i.v. BAL101553 (21.3 mg/kg, day 1), oral BAL101553 (15 mg/kg, day 1 - 5) or vehicle (buffered saline, day 1 - 5), and tumors were collected 7 days post-treatment start. Viable tumor areas

were evaluated for necrotic areas (Haematoxylin & Eosin), Ki67, and CD31. Data is represented as mean ± SD; one-way ANOVA on replicate tumor samples (n = 4); pair-wise analysis using the Tukey test.

Figure 5. Effect of BAL101553 and Bevacizumab alone and in combination on the growth of SW480 and A549EpoB40-derived tumor xenografts. (**A**) SW480- or (**B**) A549EpoB40-derived tumor xenografts were treated with vehicle or BAL101553 (21.3 mg/kg i.v. once a week or 15mg/kg p.o. daily, over three weeks) and bevacizumab (5 mg/kg on day 1 and 4 during first week) alone and in combination. (**C**) SW480derived tumor xenografts were treated with vehicle or BAL101553 (21.3 mg/kg i.v. once a week or 15mg/kg p.o. daily, over three weeks) and everolimus (5 mg/kg on day 1 – 5 during first week) alone and in combination. Each curve represents the mean tumor volume per group ± SE (n = 7-9), one-way ANOVA Tukey test.

Figure 6. Induction of tumor necrosis, reduced proliferation and enhanced anti-vascular effects with BAL101553/bevacizumab combinations in SW480 tumor xenografts. SW480-derived tumor xenografts (n = 4 per group) were treated as described, and tumors were collected 7 days post-treatment start. Mice were injected with Pimonidazole (IP; 4 μg/gm mouse weight) 1 h before tumors were harvested, formalin fixed and stained for Haematoxylin and Eosin (H&E), Ki67, CD31, Pimonidazole and Carbonic anhydrase. Scale bar: 1 mm (CD31 100 μm; 50 μm for magnified images).

Figure 7. Response to treatment with BAL101553 (21.3 mg/kg i.v. weekly or 15 mg/kg p.o. daily) and bevacizumab alone and in combination. (**A, D**) Necrotic areas (Haematoxylin & Eosin) (**B, E**) Mean vessel density (CD31) (**C, F**) % of functional perfused vessels (Ratio of Lectin+CD31+ vessels to total CD31+ vessels) in (**A, B, C**) SW480- or (**D, E, F**) A549EpoB40-derived tumor xenografts. Data is represented as mean ± SD; one-way ANOVA on replicate tumor samples (n = 4); pair-wise analysis using the Tukey test. (**G, I**) Serum levels of IL8 in murine blood derived from (**G**) SW480- or (**I**) A549EpoB40tumors treated with either bolus BAL101553 (i.v. 21.3 mg/kg, day 1) or daily BAL101553 (oral 15 mg/kg, day 1 - 5) and bevacizumab (i.p. 5 mg/kg, day 1 and 4). Tumors and blood sera were collected 7 days after treatment start; n = 4. (**H**) Representative images and quantification of immunohistochemical staining intensity for IL8 in tumor sections derived from SW480 tumors (n=4). Viable tumor sections were evaluated for specific IL8-staining intensity. Scale bar 100 μm. One-way ANOVA, data are represented as mean ± standard deviation. *P<0.05.

Supplementary Figure Legends

Suppl. Figure 1. BAL27862 exerts a broad spectrum inhibitory activity on HIF1αcontrolled secretome. In SW480 colon carcinoma cells, cancer biomarker screening was performed with Bioplex multiplex technology against conditioned media derived from untreated and BAL27862-treated cells (**A**). Cells were preincubated with BAL27862 (20nM, 24 h) followed by additional incubation for 24 hours under normoxic or hypoxic (0.2%) conditions. (**B**) Regulation of TNFα secretion by BAL27862. SW480 and A549EpoB40 cells were treated with BAL27862 (10 nM, 24 h) and cultured under normoxic and hypoxic (0.2%).

Supernatants were collected 24 hours after starting hypoxic conditions and analyzed for TNF-α protein levels. (**C**) Serum levels of TNF-α in murine blood derived from i.v. BAL101553 (21.3 mg/kg, day 1), oral BAL101553 (15 mg/kg, day 1 - 5)) or vehicle-treated SW480- and A549EpoB40-derived tumor xenografts. Tumors and blood sera were collected 7 days after treatment start.

Suppl. Figure 2. Body weight determination in response to treatment. Daily body weights of mice with SW480- **(A)** and A549EpoB- **(B)** derived tumors in response to treatment with BAL101553. Each curve represents the mean weight per group ±SEM.

Suppl. Figure 3. Combination of BAL101553 with bevacizumab in MTA-resistant tumor xenografts. Kaplan–Meier survival analysis for SW480 tumors reaching 600 mm3 tumor volume after treatment with BAL101553 (i.v. weekly or p.o. daily) or bevacizumab or their combination; n=7-9, log-rank (Mantel–Cox) test for pair-wise comparisons (A). (**B**) Representative images of immunohistochemical staining intensity for phosphorylated P70-S6kinase in tumor sections derived from SW480 tumor xenografts. (**C, D**) Daily body weights of mice with SW480- **(C)** and A549EpoB-**(D)** derived tumors in response to treatment with BAL101553 and bevacizumab. Each curve represents the mean weight per group ±SD.

Suppl. Figure 4. Induction of tumor necrosis, reduced proliferation and enhanced antivascular effects with BAL101553/bevacizumab combinations in A549EpoB40 tumor xenografts. A549EpoB40-derived tumor xenografts were treated as described and tumors were collected 7 days post-treatment start. Mice were injected with Pimonidazole (IP; 4 μg/gm mouse weight) 1 h before tumors were harvested, formalin fixed and stained for Haematoxylin and Eosin (H&E), Ki67, CD31, Pimonidazole and Carbonic anhydrase. Scale bar: 1 mm (CD31 100 μm).

Suppl. Figure 5. Immunohistochemical staining of IL8 (CXCL8) in response to combined BAL101553 and Bevacizumab regimen in A549EpoB40-derived tumor xenografts. Representative images and quantification of immunohistochemical staining intensity for IL8 in tumor sections derived from A549EpoB40 tumors (n=4). Viable tumor sections were evaluated for specific IL8-staining intensity. Scale bar 100 μm. One-way ANOVA, data are represented as mean ± standard deviation. *P<0.05.

Conflict of Interest statement

F. Bachmann and H. A. Lane are employees of Basilea Pharmaceutica International Ltd and shareholders of Basilea Pharmaceutica Ltd.

Acknowledgement

The study was supported by research grants of Basilea Pharmaceutica International Ltd. and Krebsforschung Schweiz (to M.P.). Tamara Kazimova is an early stage researcher and Martin

Pruschy is a beneficiary in the RADIATE-ITN and as such have received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement No 642623.

Figure 1

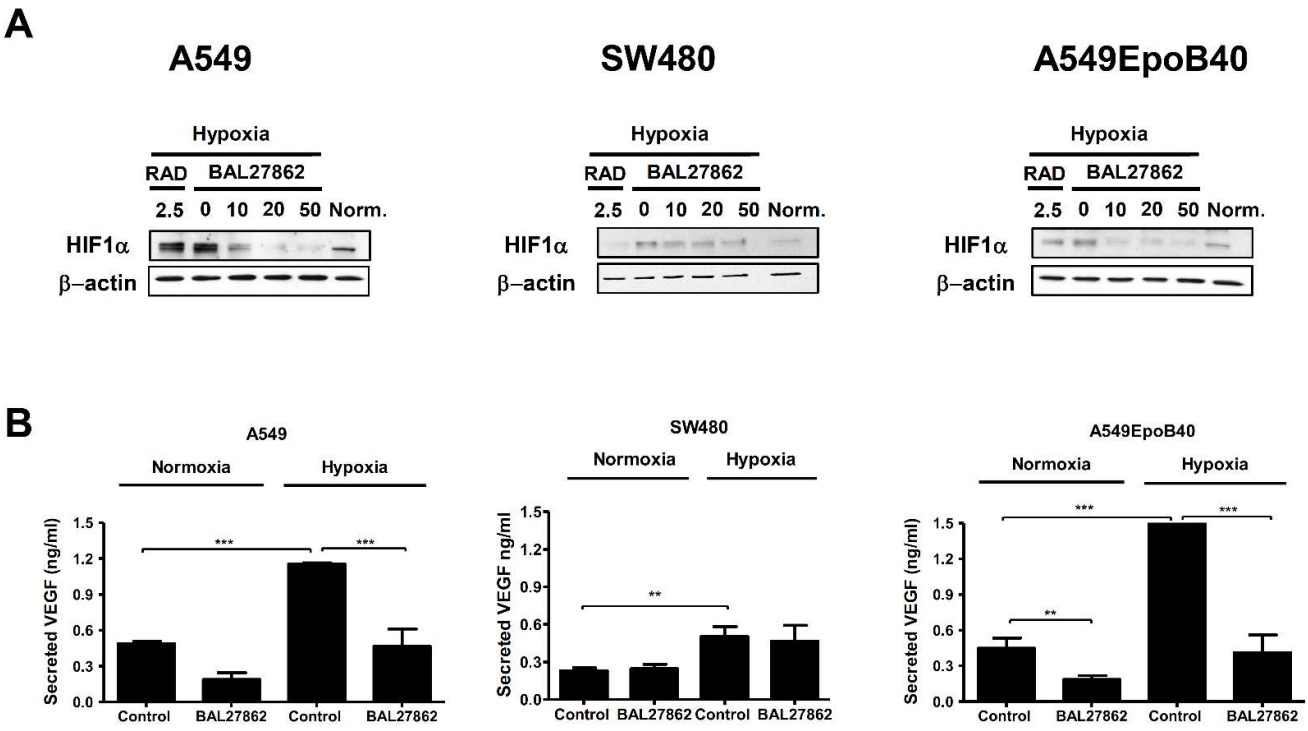
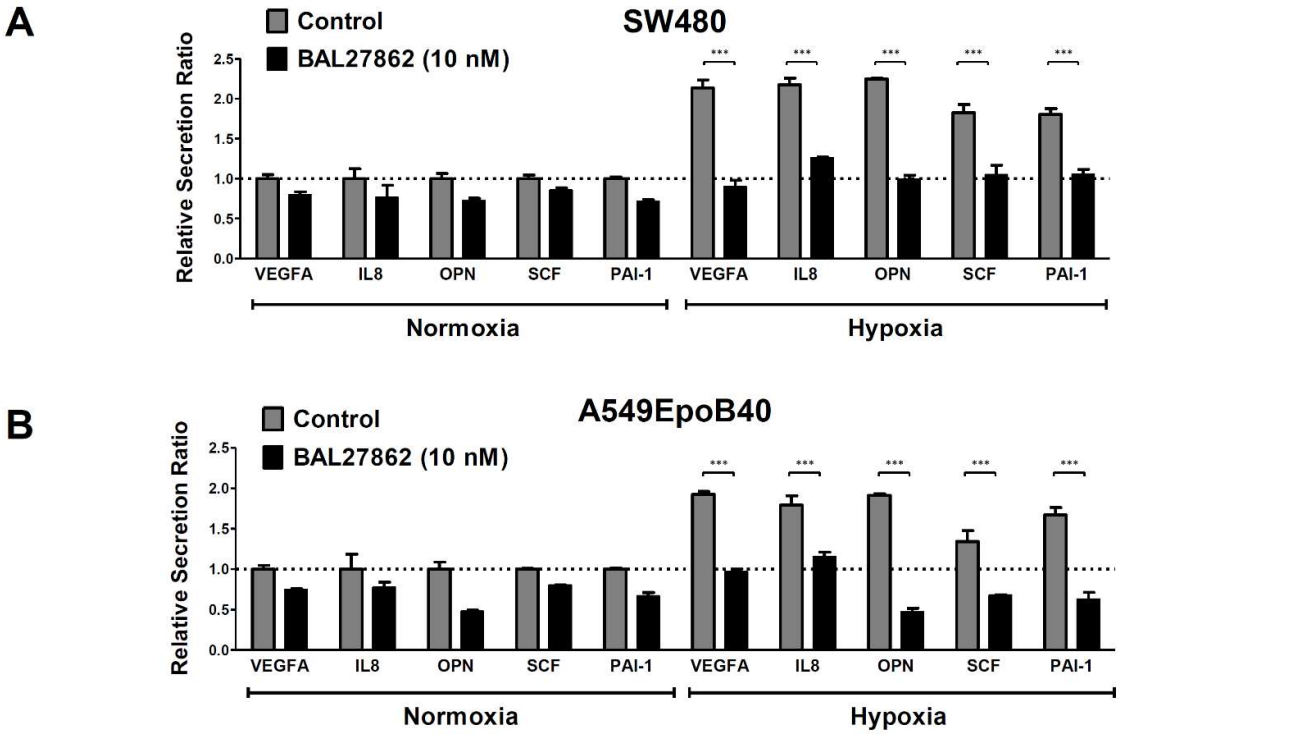
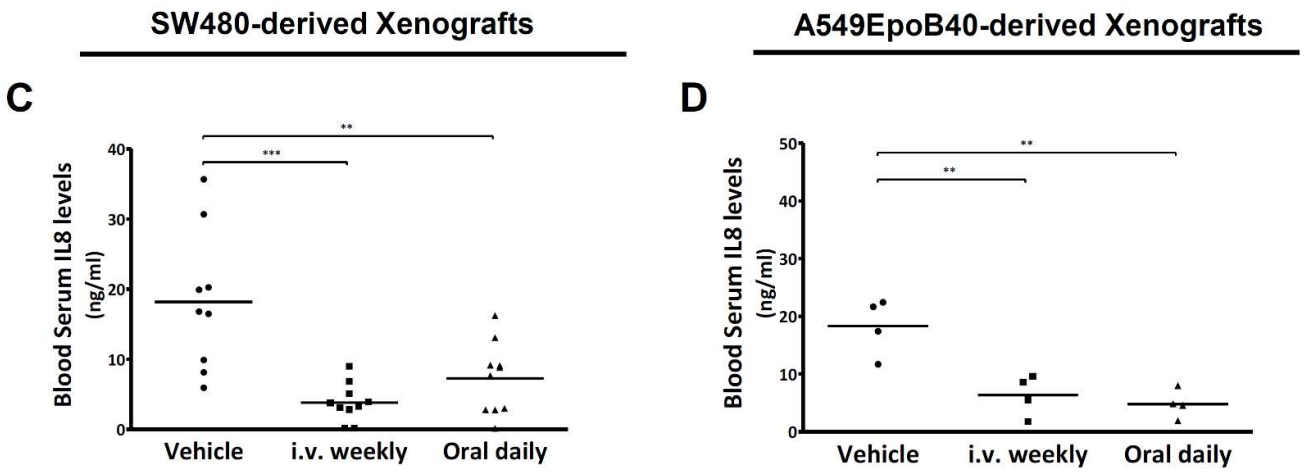

A
A549
SW480
A549EpoB40
Hypoxia
RAD
BAL27862
2.5 0 10 20 50 Norm.
HIF1α
β–actin
B
A549
SW480
A549EpoB40
Normoxia
Hypoxia
Secreted VEGF (ng/ml)
Secreted VEGF ng/ml
***
**
Control
BAL27862

Figure 2


A
Control
BAL27862 (10 nM)
SW480
Relative Secretion Ratio
VEGFA
IL8
OPN
SCF
PAI-1
Normoxia
Hypoxia
B
A549EpoB40


SW480-derived Xenografts
A549EpoB40-derived Xenografts
C
D
Blood Serum IL8 levels (ng/ml)
Vehicle
i.v. weekly
Oral daily

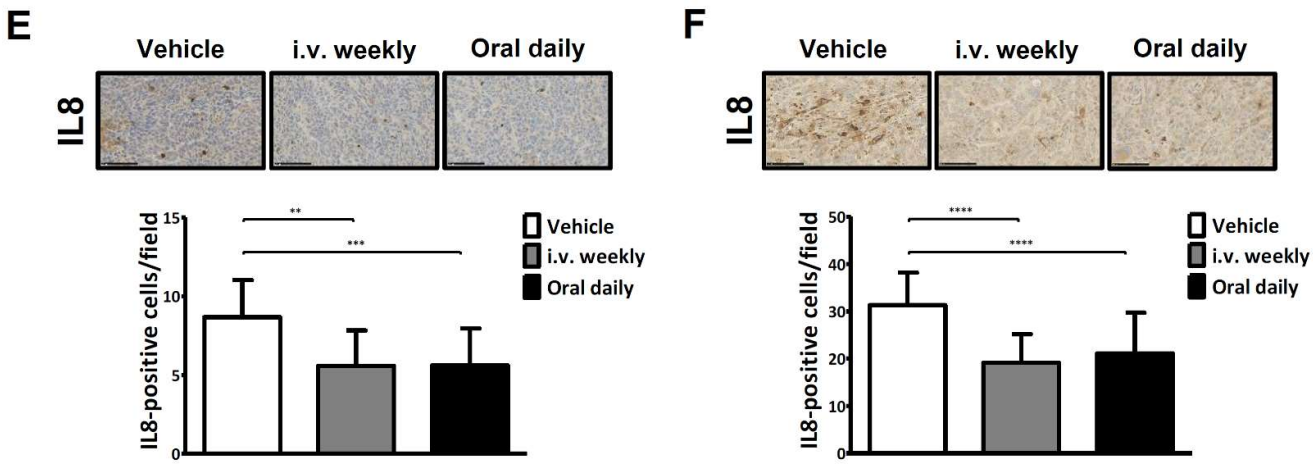
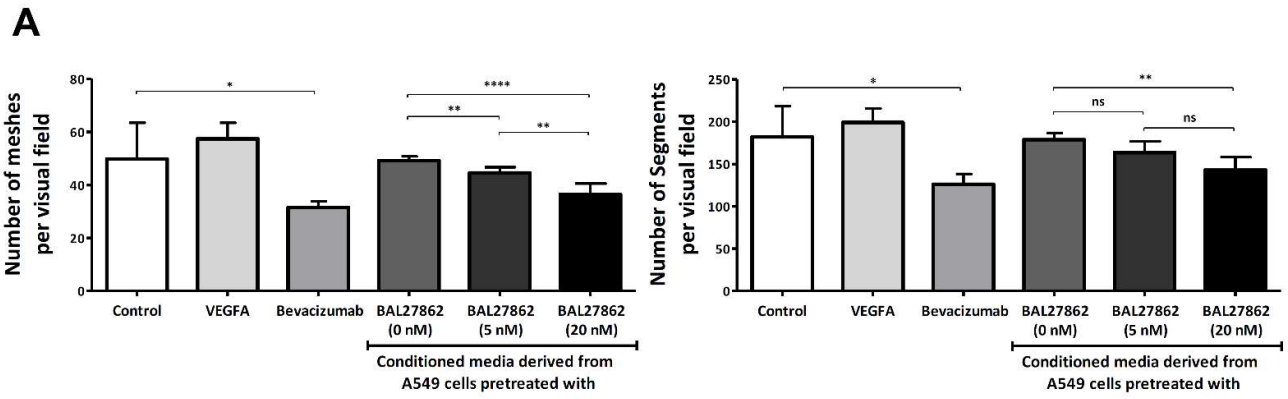
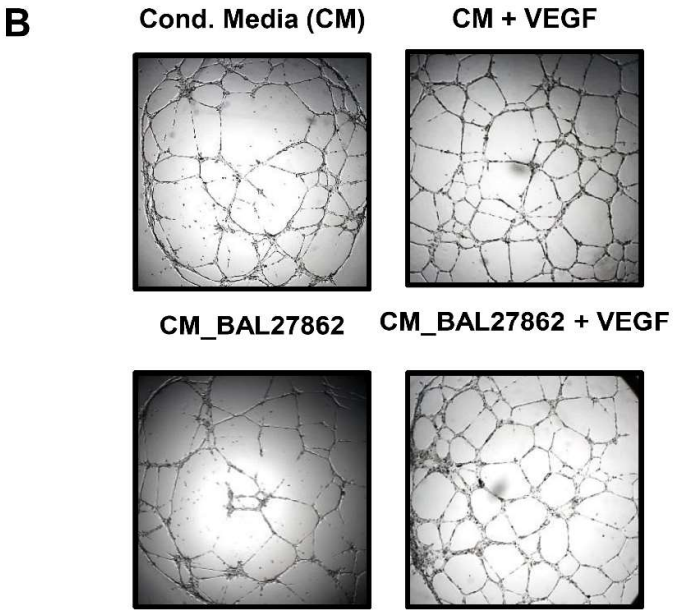

E
F
IL8
Vehicle
i.v. weekly
Oral daily
IL8-positive cells/field

Figure 3


A
Number of meshes per visual field
Number of Segments per visual field
Control
VEGFA
Bevacizumab
BAL27862 (0 nM)
BAL27862 (5 nM)
BAL27862 (20 nM)
Conditioned media derived from A549 cells pretreated with
ns


B
Cond. Media (CM)
CM + VEGF
CM_BAL27862
CM_BAL27862 + VEGF

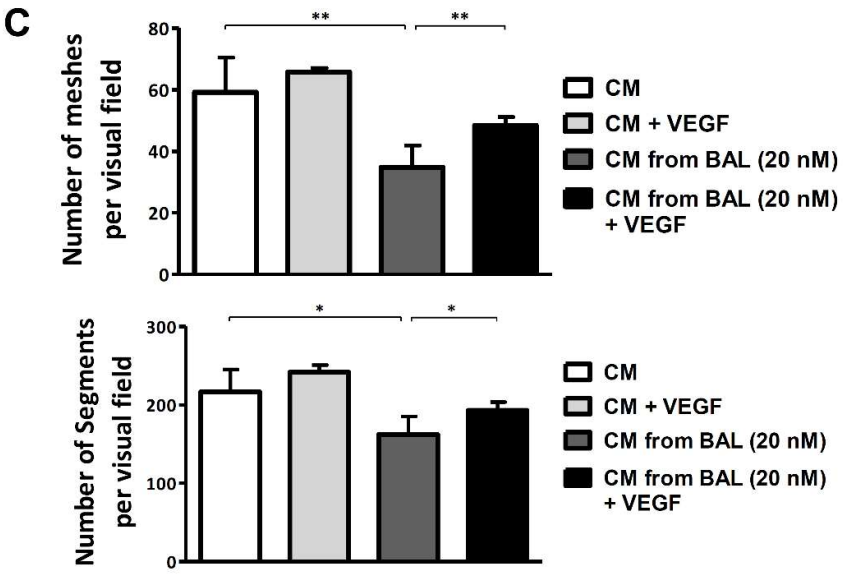

C
Number of meshes per visual field
Number of Segments per visual field
CM
CM + VEGF
CM from BAL (20 nM)
CM from BAL (20 nM) + VEGF

Figure 4

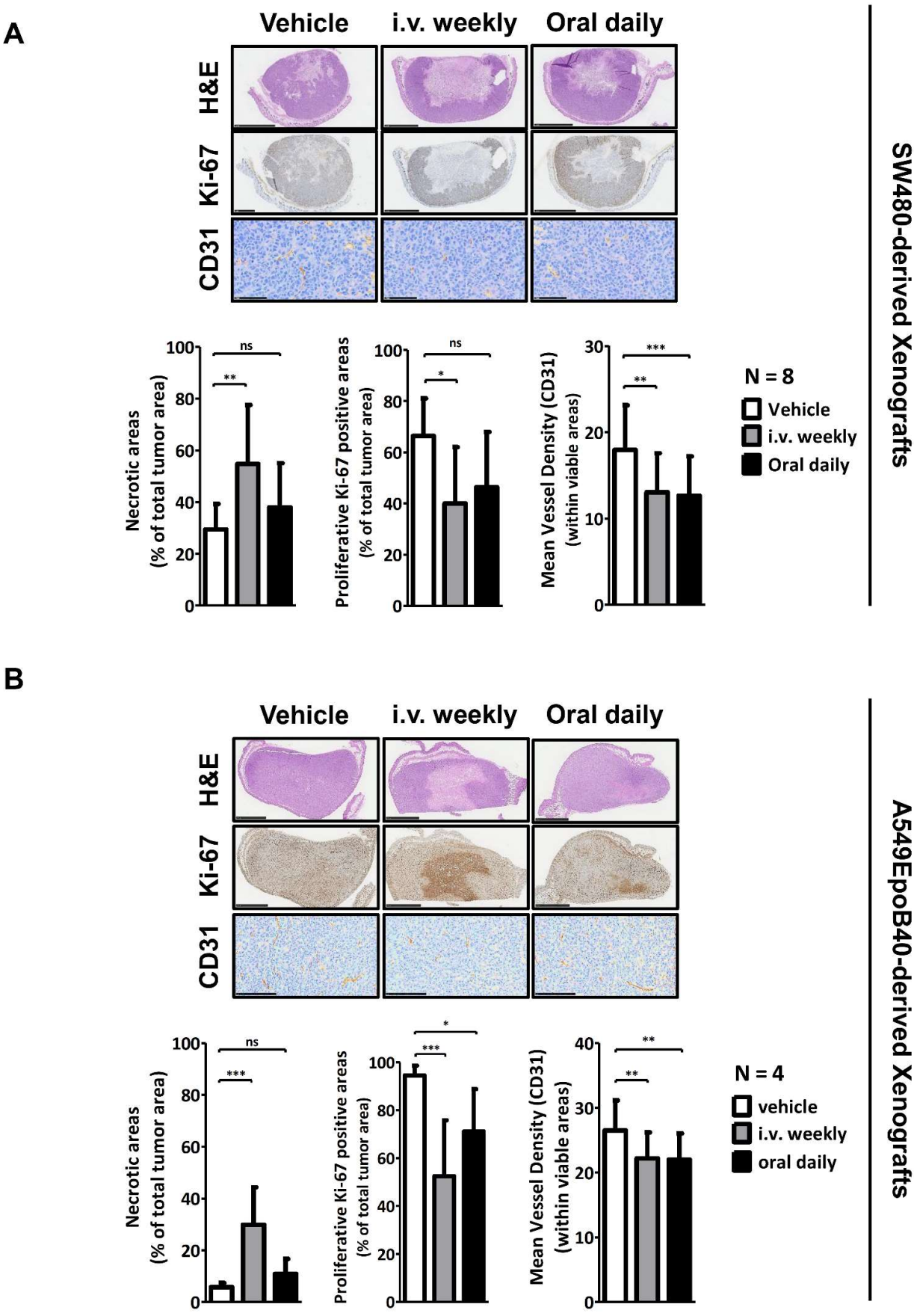

A
Vehicle
i.v. weekly
Oral daily
H&E
Ki-67
CD31
Necrotic areas (% of total tumor area)
Proliferative Ki-67 positive areas (% of total tumor area)
Mean Vessel Density (CD31) (within viable areas)
ns
**
*
***
N = 8
Vehicle
i.v. weekly
Oral daily
SW480-derived Xenografts
B
Vehicle
i.v. weekly
Oral daily
H&E
Ki-67
CD31
Necrotic areas (% of total tumor area)
Proliferative Ki-67 positive areas (% of total tumor area)
Mean Vessel Density (CD31) (within viable areas)
ns
***
*
**
N = 4
vehicle
i.v. weekly
oral daily
A549EpoB40-derived Xenografts

Figure 5

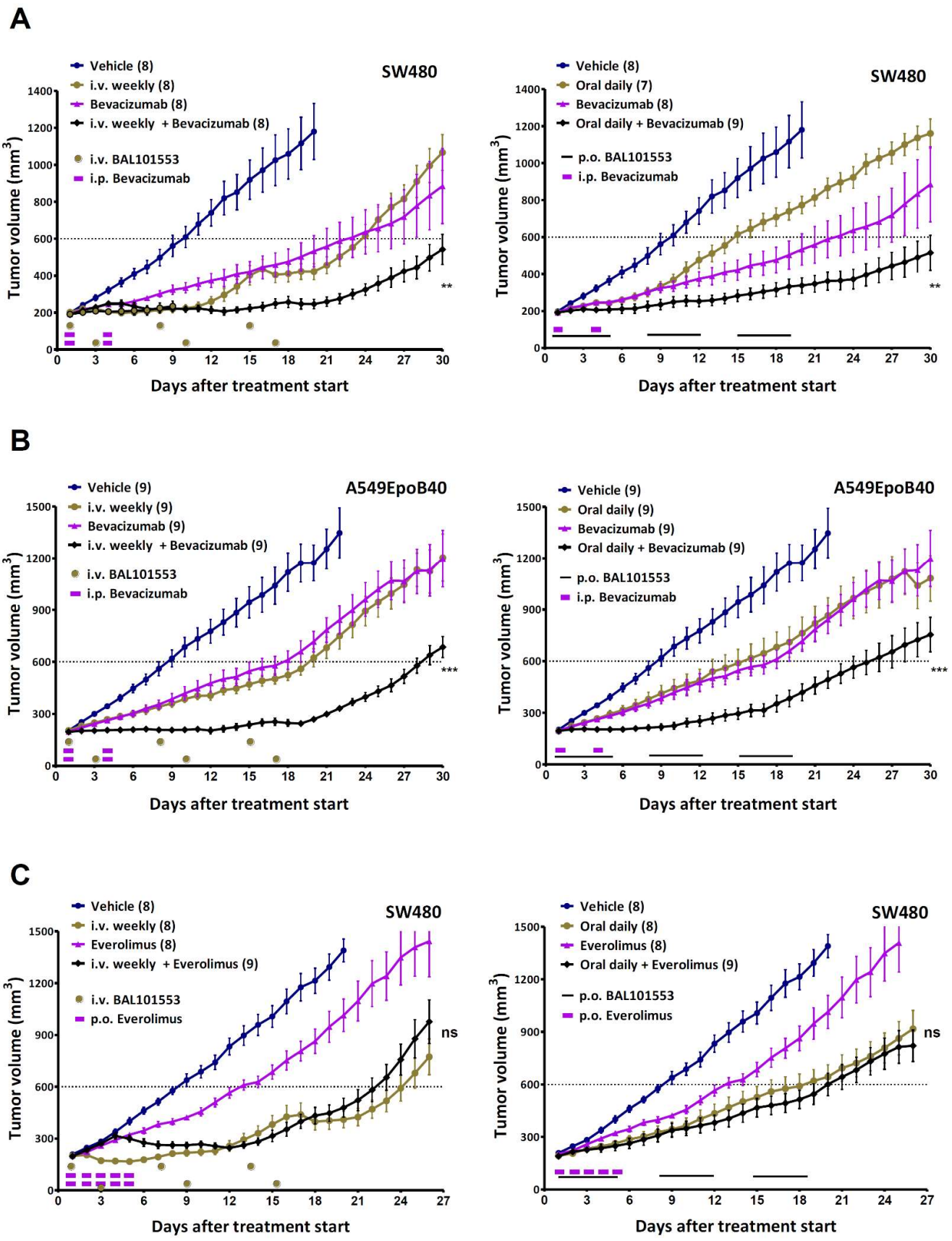

A
Vehicle (8)
i.v. weekly (8)
Bevacizumab (8)
i.v. weekly + Bevacizumab (8)
i.v. BAL101553
i.p. Bevacizumab
SW480
Tumor volume (mm3)
Days after treatment start
**
Vehicle (8)
Oral daily (7)
Bevacizumab (8)
Oral daily + Bevacizumab (9)
p.o. BAL101553
i.p. Bevacizumab
SW480
Tumor volume (mm3)
Days after treatment start
**
B
Vehicle (9)
i.v. weekly (9)
Bevacizumab (9)
i.v. weekly + Bevacizumab (9)
i.v. BAL101553
i.p. Bevacizumab
A549EpoB40
Tumor volume (mm3)
Days after treatment start
***
Vehicle (9)
Oral daily (9)
Bevacizumab (9)
Oral daily + Bevacizumab (9)
p.o. BAL101553
i.p. Bevacizumab
A549EpoB40
Tumor volume (mm3)
Days after treatment start
***
C
Vehicle (8)
i.v. weekly (8)
Everolimus (8)
i.v. weekly + Everolimus (9)
i.v. BAL101553
p.o. Everolimus
SW480
Tumor volume (mm3)
Days after treatment start
ns
Vehicle (8)
Oral daily (8)
Everolimus (8)
Oral daily + Everolimus (9)
p.o. BAL101553
p.o. Everolimus
SW480
Tumor volume (mm3)
Days after treatment start
ns

Figure 6.

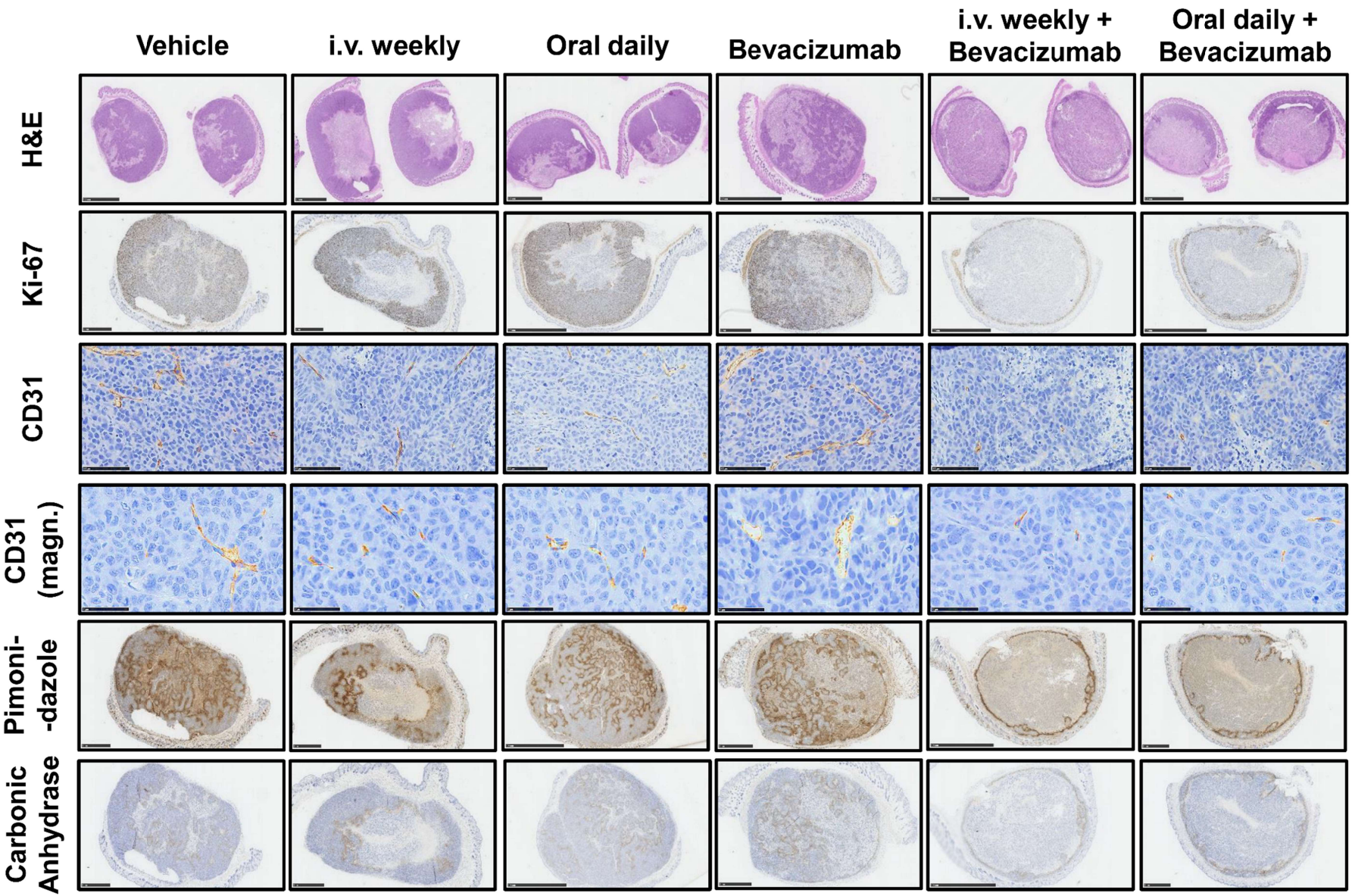
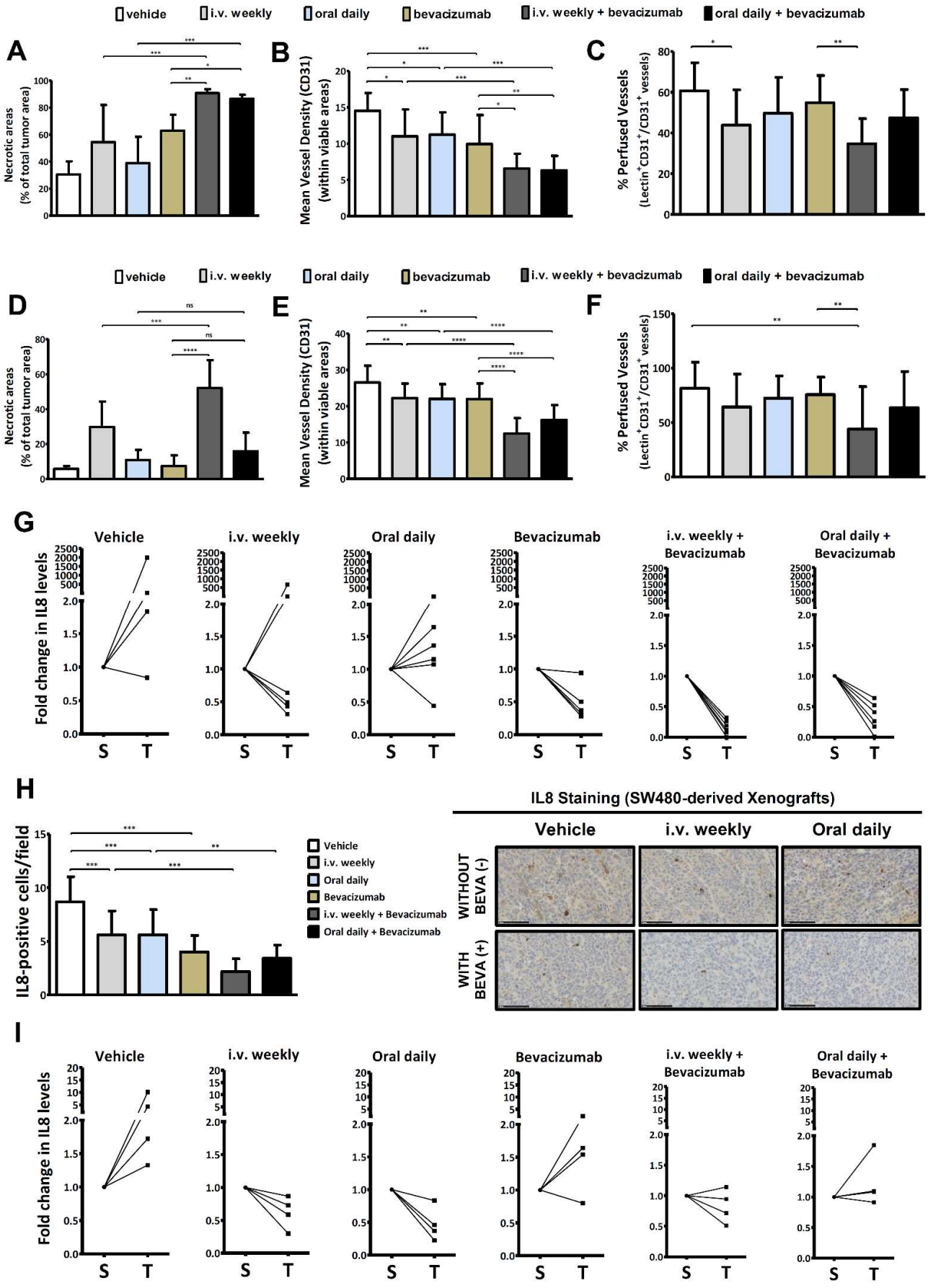
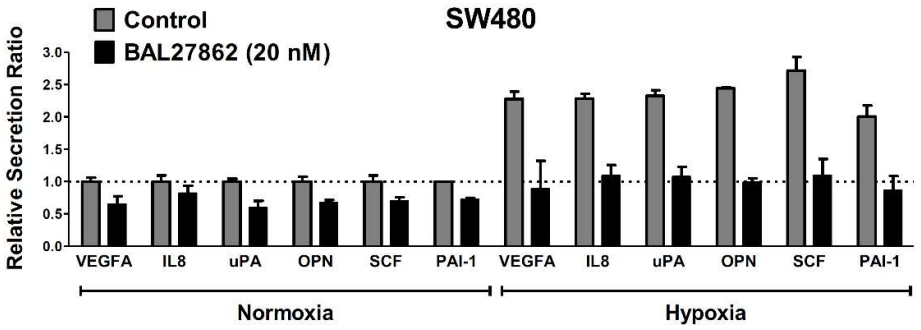

Vehicle
i.v. weekly
Oral daily
Bevacizumab
i.v. weekly + Bevacizumab
Oral daily + Bevacizumab
H&E
Ki-67
CD31
CD31 (magn.)
Pimoni-dazole
Carbonic Anhydrase

Figure 7

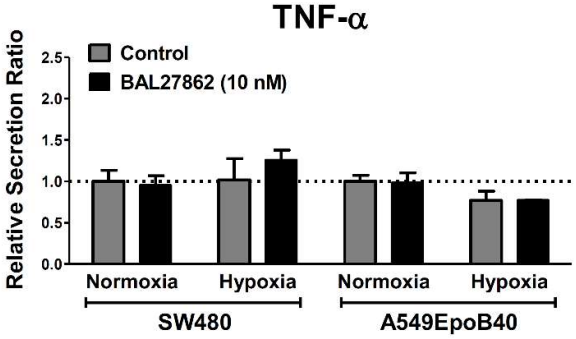

vehicle
i.v. weekly
oral daily
bevacizumab
i.v. weekly + bevacizumab
oral daily + bevacizumab
A
B
C
Necrotic areas (% of total tumor area)
Mean Vessel Density (CD31) (within viable areas)
% Perfused Vessels (Lectin+CD31+/CD31+ vessels)
D
E
F
G
Vehicle
i.v. weekly
Oral daily
Bevacizumab
i.v. weekly + Bevacizumab
Oral daily + Bevacizumab
Fold change in IL8 levels
S
T
H
IL8-positive cells/field
IL8 Staining (SW480-derived Xenografts)
WITHOUT BEVA (-)
WITH BEVA (+)
I

Supplementary Figure 1

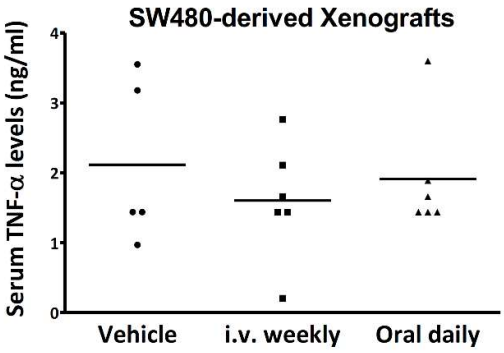
A

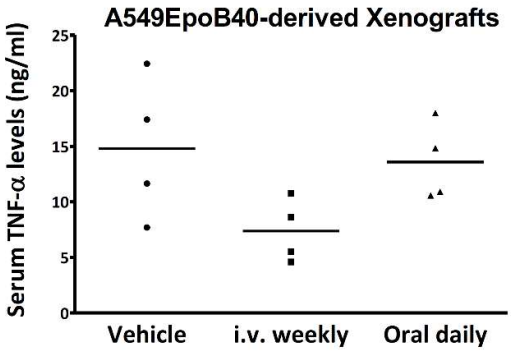


B



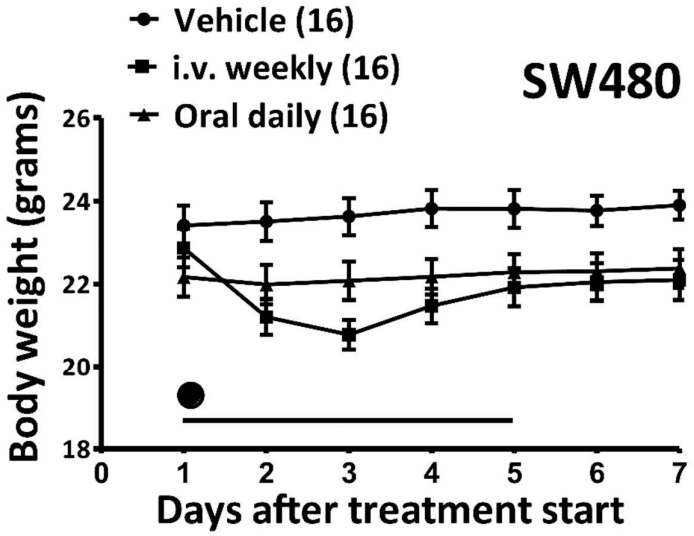
C



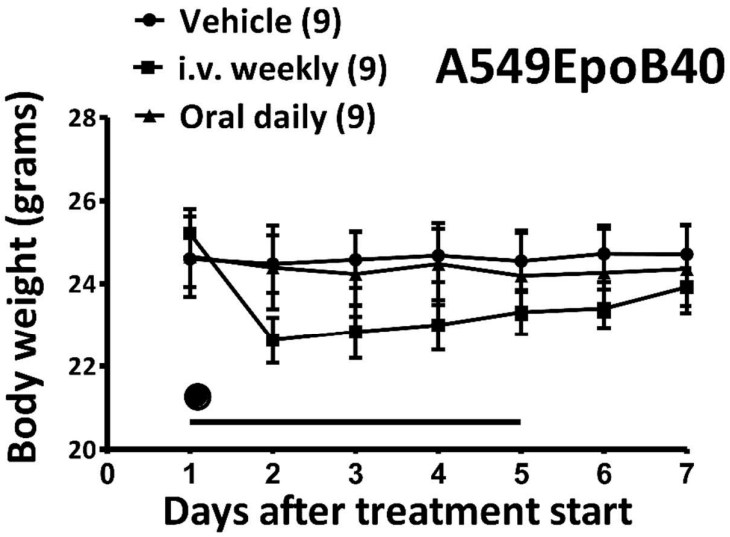


Supplementary Figure 2

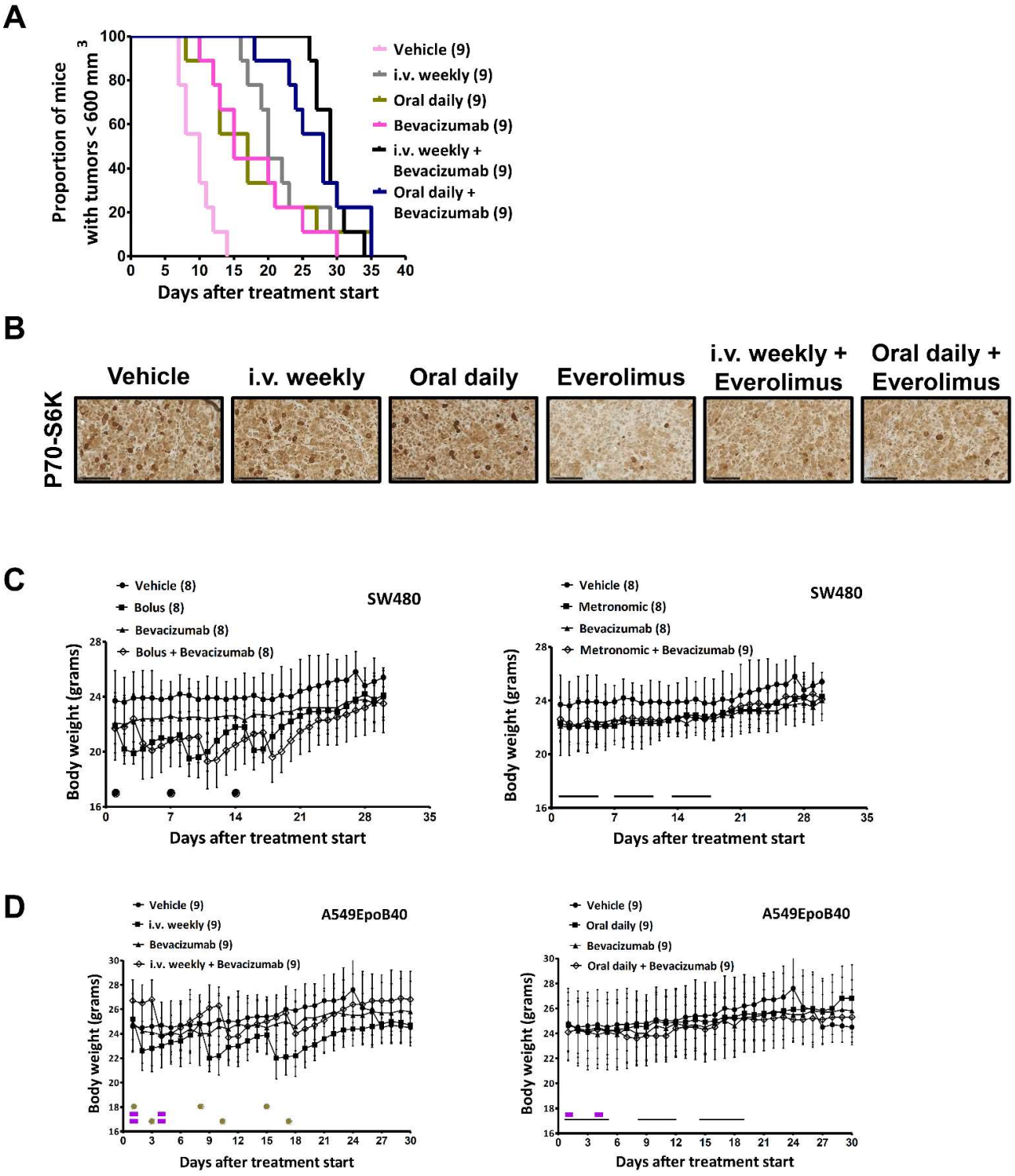
A



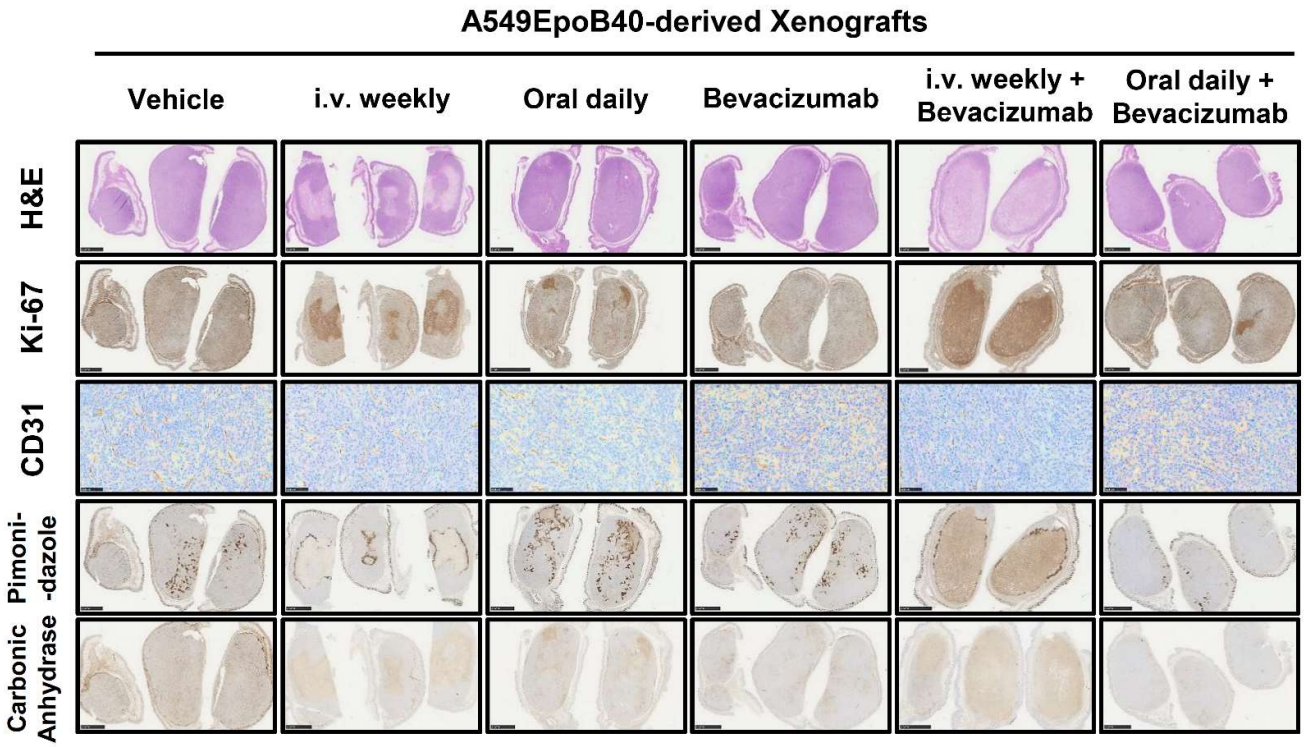
B



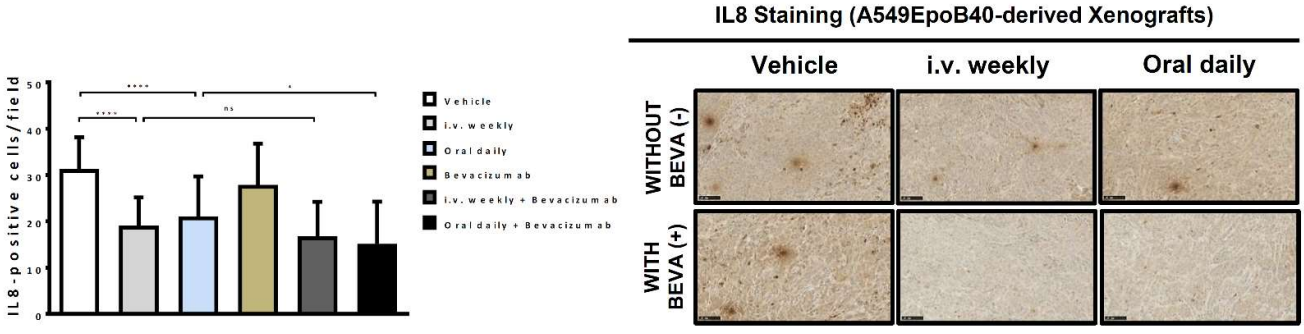
Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5



3.2 Placental growth factor regulation in p53 wild-type tumors in response to ionizing radiation

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Status of the manuscript: Ready for submission

Author contribution Tamara Kazimova

- Planning, data acquisition, analysis and interpretation of the experiments
- Figure 1 – B – E
- Figure 2 – all
- Figure 3 – all
- Figure 4 – all
- Figure 5 – all
- Figure 6 – all
- Writing of the manuscript

Placental growth factor regulation in response to ionizing radiation is p53dependent

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Running title: Placental growth factor and ionizing radiation

Keywords: placental growth factor, p53, ionizing radiation, hypoxia

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Disclosure of Potential Conflicts of Interest: No potential conflicts of interest exist.

Word count (excluding references and figure legends): 4921

Total number of figures and tables: 7

Abstract

Purpose: Placental growth factor (PlGF) is a pro-angiogenic, N-glycosylated and secreted growth factor. As a stress response to ionizing radiation (IR), tumor cells show increased secretion of PlGF. Here, we investigated the regulation of PlGF in response to IR and its role for tumor angiogenesis and radiosensitivity.

Experimental design: Secretion and expression kinetics of PlGF across multiple cancer cell lines was determined using ELISA. PlGF regulation was mechanistically investigated in D341, DAOY and HCT116 cells. Tumor angiogenesis and radiosensitization in PlGF wildtype and PlGF knockout cells were determined in a medulloblastoma tumor xenograft model.

Results: Secretion and expression of PlGF in response to irradiation was investigated in multiple tumor cell lines in a dose- and time-dependent manner. IR induced early upregulation of PlGF expression in p53 wildtype tumor cells, whereas tumor cells with mutated p53 only showed a minimal or delayed response. Mechanistic investigations with genetic and pharmacological targeting of p53 corroborated regulation of PlGF by the tumor suppressor p53. A paracrine role of PlGF was investigated by exploring migration of irradiated HUVECs towards non-irradiated and irradiated PlGF wildtype and PlGF knockout medulloblastoma cells and tumor xenografts thereof. Tumors derived from PlGF-ko cells displayed a reduced growth rate, but similar tumor vasculature formation as their wildtype counterparts. Interestingly though, high-dose irradiation strongly reduced microvessel density with a concomitant high rate of complete tumor regression only in the PlGF-ko tumors.

Conclusions: Expression and secretion of PlGF in response to irradiation is strongly regulated by transcriptional activity of the tumor suppressor p53. In p53-mutated tumor cells though, hypoxia-related factors and other so far unresolved mechanisms strongly contribute to a delayed expression of PlGF. Irradiation-induced PlGF plays a strong vasculature-protective role thereby contributing to enhanced radiation resistance in vivo. These results suggest PlGF to be a relevant target for a combined treatment modality with radiotherapy.

Introduction

Radiotherapy (RT) is widely used as part of combined treatment modality and is considered as one of the most effective treatments for the management of solid tumors. In addition to DNA damage and genome instability, ionizing radiation (IR) also leads to stress responses in tumor cells by activating signal transduction pathways and inducing secretion of several auto- and paracrine factors [1, 2]. IR is usually combined with pharmaceutical agents targeting biologically relevant resistance mechanisms to achieve local tumor control and improve survival rates [3-7]. We recently performed an exhaustive semi quantitative dot blot secretome analysis from irradiated tumor cells and identified among others that placental growth factor (PlGF) is secreted from tumor cells in response to irradiation. A secondary analysis revealed that multiple of these factors, but not PlGF, are substrates of the matrix metalloproteinase ADAM17 and that irradiation enhances its sheddase activity in an IR-dose-dependent way [8].

PlGF is an N-glycosylated, homodimeric protein and belongs to the vascular endothelial growth factor (VEGF)-family, with 53% similarity to the platelet derived growth factor (PDGF)-like region of VEGF [9]. The PlGF gene contains seven exons and due to alternative splicing exists in four isoforms consisting of 131, 152, 203 and 224 amino acids [10, 11]. Under normal conditions, PlGF expression is low in organs such as heart, skeletal muscle and lungs [12-14]. Binding of PlGF to its receptor vascular endothelial growth factor receptor 1 (VEGFR1) leads to phosphorylation of the receptor, activation of downstream pro-angiogenic signaling pathways and a crosstalk between VEGFR-1 and 2 [15-18]. Compared to VEGF-A, PlGF is not as extensively investigated and its regulation and role in response to irradiation is poorly understood. PlGF is highly expressed in the placenta, however, its deletion does not affect embryonic development in mice [16]. While PlGF-deficient mice can develop normally, they are unable to adapt to pathological conditions such as inflammation or cancer [16, 17].

Several studies demonstrate the contribution of PlGF to tumor angiogenesis under pathological conditions. Tumor stage, metastasis and poor overall survival correlate to increased PlGF levels in different tumors entities [19-24]. Of note inhibition of VEGF and its receptors leads to increased PlGF levels, probably contributing to escape and resistance against these treatment modalities [24-27]. On the preclinical level as

investigated in several mouse models, blocking of PlGF by RNA interference, neutralizing antibodies or gene silencing resulted in decreased angiogenesis, reduced tumor growth and dissemination [16, 24, 28-32]. Interestingly, while blockage of PlGF normalized tumor vessels in some tumor models [28, 33], tumor vessel normalization was also observed in PlGF overexpressing tumors [34].

Expression of PlGF is regulated by several transcription factors, however contradictory results exist on the regulation of PlGF by the tumor suppressor p53 and the hypoxiainducible factor HIF-1α [9, 16, 18, 29, 35-37]. Furthermore, almost no data exist on the regulation of PlGF in response to irradiation [38]. Here we investigate expression and secretion of PlGF across multiple tumor cell lines in response to irradiation and in dependence of the tumor suppressor protein p53. Short-time efficacy-oriented experiments performed in vivo indicate a vessel-protective role of PlGF in response to irradiation.

Materials and methods

Cell cultures and treatments

Human lung adenocarcinoma cells (A549, H460, H358, H292, H125) (ATCC) were cultured in RPMI1640 media, medulloblastoma cells (D341, D425, DAOY, UW228) (kindly provided by Martin Baumgartner, Kinderspital) in improved MEM media (Richter's modification) and head and neck squamous cell carcinoma cells (FaDu) (ATCC) in DMEM media. All media were supplemented with 10% (v/v) fetal calf serum, 1% (v/v) penicillin-streptomycin and 1% (v/v) L-glutamine and cells were kept at 37 °C in 5% CO_2. All cell culture media and supplements were purchased from Gibco (Life Technologies). HUVECs, Endothelial Cell Growth Medium (ECGM) and supplementary growth factors were purchased from Promocell. Cells were pretreated with Pifithrin-α (S2929, Selleckchem), MI-773 (S7649, Selleckchem), BAY 87-2243 (S7309, Selleckchem) in given concentrations 1-2 hours prior to irradiation with an Xstrahl 200kV X-ray unit at 1Gy/min or RS-2000 225kV irradiator at 4.2Gy/min (Rad Source). Hypoxia experiments were performed at 1% O_2 after cells were treated with pharmacological agents and irradiated.

Bio-Plex assay

Bio-Plex (Bio-Rad, 6156 and 6304) is a fluorescently dyed bead-based assay, with similar principles to a sandwich ELISA, where each bead has a color code permitting detection of different biomarkers in a suspension. Briefly, beads are covalently coupled to a capture antibody directed against specific biomarker and are incubated with standards or samples (supernatant derived from non-irradiated and irradiated A549 cells). After a washing step, beads are incubated with biotinylated detection antibodies. The unbound biotinylated antibodies are washed and the beads are incubated with a fluorescence reporter streptavidin-phycoerythrin conjugate (SA-PE), forming the detection complex. After an additional washing step, the fluorescence of each bead with bound SA-PE was measured when the beads were passed through the two laser Bio-Plex reader. The lasers provide bead classification and a reporter signal from PE. All incubations were carried out at room temperature. The fluorescence signal is detected by photomultiplier tube (PMT). The data are then presented as median fluorescence intensity (MFI) and concentration (pg/ml) using a Bio-Plex Manager™ software, where concentration is proportional to MFI of the reporter.

ELISA

Secreted PlGF concentration was detected in filtered conditioned media 24 hours and 48 hours after irradiation using a PlGF DuoSet ELISA kit according to manufacturer's guidelines (R&D Systems). The absorbance was determined with plate reader EL808 Ultra Microplate Reader (Bio – Tek Instruments, Inc.) at 450nm excitation and 570nm emission, and normalized to live cell count using EVE Automatic cell counter (NanoEnTek).

qRT-PCR

Sample mRNA expression was determined at 4 hours and 24 hours after irradiation. Cell lysate collection and RNA isolation were performed using an RNeasy Mini Kit (Qiagen) according to manufacturer's guidelines. RNA was reverse-transcribed (High Capacity cDNA Reverse Transcription Kit from Applied Bioscience) and cDNA was amplified using SYBR Green Master Mix (Roche) and the following primers: (5'-3'): GAPDH forward: AACGGATTTGGTCGTATTGGGC; GAPDH reverse: TTGATTTTGGAGGGATCTCG; PlGF forward: TGTCACCATGCAGCTCCTAA; PlGF reverse: AGCATCGCCGCACCTTTC; p53 forward: CCCTTCCCAGAAAACCTA; p53

reverse: CTCCGTCATGTCCTGTGA. qRT-PCR was performed on LightCycler 480 (Roche).

siRNA transfection

Transfection was performed by using reverse transfection with Lipofectamine RNAiMAX (Invitrogen) in antibiotic-free medium. Cells were re-seeded into 6-well plates 24h later with fresh medium supplemented with antibiotics. siLuc was synthesized by Microsynth with the following sequence: (5'-3'): CGUACGCGGAAUACUUCGATT. sip53 was purchased as a pool of four siRNAs from Dharmacon. Irradiation was performed 48 hours after siRNA transfection.

Western Blotting

Whole cell extracts were collected using Laemmli buffer and samples were boiled for 5 minutes at 95 °C. 40-50µg of protein were separated on 10-15% SDS-PAGE gel and transferred to PVDF membrane. The membrane was then blocked in 5% non-fat milk and incubated with primary antibodies (rabbit polyclonal anti-p53 (GTX102965, GeneTex), rabbit monoclonal anti-p21 (2947S, Cell Signaling), rabbit monoclonal anti-HIF-1α (D2U3T, Cell Signaling), mouse monoclonal anti-β-actin, (A1978, Sigma)) at 4°C overnight. After three washings, HRP conjugated secondary antibodies (mouse anti-rabbit (SC-2357, Santa Cruz) or sheep anti-mouse (NA931V, GE Healthcare)) were added for one hour at room temperature followed by additional washing steps. The membrane was developed with the ECL system (Amersham Bioscience) according to manufacturer's protocol in Fusion FX (Vilber).

Genomic deletion of PlGF via CRISPR/Cas9 in D341 cells

Two separate sgRNAs targeting different regions of the third exon of PGF gene (target sequence 1: GAATCTGCACTGTGTGCCGG; target sequence 2:
CGTGTCCGAGTACCCCAGCG) were generated using freely available online tools (UCSC and CRISPOR) and the primers were synthesized by Microsynth. The singlestranded oligomers were annealed and ligated into GFP and Cas9 expressing pLentiCRISPR-EGFP plasmid (cat. nr. 75159, Addgene, kindly provided by Beat Schäfer) using the Golden Gate assembly cloning strategy. The ligation mix was transformed into competent bacteria by heat shock. Clones with pLentiCRISPR plasmid was identified by colony-PCR using an U6 promoter specific forward primer (5' –GACTATCATATGCTTACCGT- 3'). Positive colonies were inoculated for midiprep

(Sigma) culture. HEK293T (ATCC) cells were used for production of lentiviral constructs according to guidelines from Cellecta. Filtered lentiviral supernatant was used to transfect the target cells (D341). To prepare cells for FACS, live cells were harvested, re-suspended in PBS with 2% FBS and filtered using a 40µm cell strainer (BD Falcon). Cell sorting was performed with a FACSAria^{TM} III Cytometry System (BD Biosciences*).* Sorted cells were further subcloned through limited dilution and sequenced before further experiments. Each subclone was named after their respective well number, i.e. control cell lines C4, C6, and knock out cell lines B5, B7, G7and F8.

Migration Assay

For the transwell migration assay, 24-well transwell units (6.5mm) with 1µm pore size PET membranes (Greiner Bio-One, 662610) were used according to the manufacturer's instructions. Briefly, $3x10^5$ attracting cells (D341 PlGF-wt or PlGF-ko) were plated into the lower chamber of the transwell containing 1000µl supplemented with 1% FBS and without growth factors and allowed to attach for a minimum of 6h. The plate was sham or 5Gy irradiated. Next, $3x10^4$ endothelial cells in 200µl of the same ECGM medium as above were seeded into the upper chamber of the transwell inserts and allowed to attach for a minimum of 6h. Thereafter, the inserts were irradiated with 5Gy and immediately placed on the wells harboring the attracting cells. The co-culture was maintained at 37°C in 5% CO_2 for 48 hours. For quantification, cells from the upper side of the insert were scraped away with a cotton swap and inserts were then fixed in Methanol/Acetic Acid (75%/25%, v/v), dried and stained with DAPI (1:25000) in 99% MetOH. Fluorescent microscopy pictures were taken (Leica 7000DT) and the migrated cells were counted manually in at least 3 images at 20x magnification per insert.

In vivo experiments

D341 PlGF control and PlGF knockout subclones were injected subcutaneously in the back of 8-week old, female athymic CD1 nude mice (Charles River) with 4 x 10^6 cells/150 µl. Tumor volumes were determined with caliper according to the formula (L x l^2)/2. Treatment started when tumors reached a volume of 150-200mm^3±10%. Tumors were sham-irradiated or irradiated with a single dose of 5 or 10Gy for tumor histology using an image-guided small animal radiotherapy platform (Precision X-Ray, X-Rad SmART) 225kV unit with a dose rate of 3Gy/min, equipped with a cone beam

CT (CBCT) scanner. Precise irradiation plans were designed with the corresponding SmARTPlan software. Radiation therapy was applied with two opposing fields. Animals were kept under 3% isoflurane anesthesia for imaging and treatment. 8 days after treatment, mice were euthanized in a CO_2 chamber, tumors were harvested and immediately fixed in formalin. All in vivo experiments were performed according to guidelines for the welfare and use of animals of the Veterinäramt Kanton Zurich, Switzerland.

Immunohistochemistry

Immunohistological endpoints were analyzed on formalin-fixed paraffin-embedded

(FFPE) 4µm tissue sections derived from PlGF control or PlGF ko-derived tumor xenografts for hematoxyline and eosine (H&E), smooth muscle actin (SMA) (1:50, Dako, M0851) and CD31 (1:10, Dako, M0823). Images were taken on a wide-field Nikon Eclipse TI microscope. Amount of vessels were counted in at least 10 different fields in each xenograft.

Statistical analysis

Experiments were performed at least three times and data were analyzed using the unpaired Student *t test* and one-way ANOVA with post Tukey test using GraphPad Prism 5. Error bars indicate SEM. A p-value ≤ 0.05 was considered significant.

Results

Ionizing radiation increases PlGF expression and secretion across multiple cancer cell lines

Secretome analysis by semiquantitative antibody array screening previously indicated that PlGF is secreted in response to irradiation [8]. Additional bioplex-based quantification of 32 major cancer biomarkers in the supernatant from irradiated A549 lung adenocarcinoma cells now revealed that PlGF is most strongly increased in response to irradiation (Figure 1A). In order to analyze irradiation-induced PlGFexpression in more detail and across additional tumor cell lines derived from different tumor entities (colon, medulloblastoma, lung carcinoma), HCT116-, D341-, DAOY, A549-, and H125 carcinoma cells were irradiated and supernatants thereof analyzed for PlGF secretion 24 and 48 hours after irradiation. IR-enhanced PlGF-secretion was identified in the conditioned media (CM) from all cell lines by ELISA in a time- and IRdose-dependent manner (Figure 1B-F).

Enhanced secretion in response to irradiation might be related to IR-induced PlGF-expression. Upregulation of PlGF expression was observed in several cancer cell lines (e.g. HCT116, A549, D341) as early as 4 hours after irradiation (Figure 2), while only minimally or only observed in a delayed way in other cell lines (e.g. H358, FaDu, UW228) 24 hours after irradiation (Supplementary figure 1). Based on a literature search on the mutational status of Kras and p53 in these cell lines (Supplementary Table 1), this differential IR-induced expression pattern of PlGF could be correlated with the mutational status of the tumor suppressor and transcription factor p53.

Interestingly, cells expressing wildtype p53 demonstrated IR-induced PlGF expression as early as 4 hours after irradiation, which correlated with early PlGF secretion. In contrast cells expressing mutant p53 did not show any changes in the PlGF expression level at this early time point after irradiation, suggesting that p53 regulates IR-induced PlGF expression. Of note, delayed expression of PlGF in the p53 mutated cells in response to irradiation correlated with delayed PlGF secretion into the CM of the respective cells (48 hours' time point) (see above Figure 1).

Due to their high PlGF expression and secretion levels, and that treatment of medulloblastoma patients includes radiotherapy [39], further experiments were primarily performed with p53 wildtype D341 cells.

p53 is the main regulator of PlGF in p53 wildtype cancer cells

To determine regulation of PlGF-expression and secretion by p53, D341 and HCT116 cells were treated with increasing concentrations of the MDM2-inhibitor MI-773. MDM2 downregulates the transcriptional activity of p53 by direct binding to the tumor suppressor p53 and by ubiquitination and inducing its proteasomal degradation [40]. Thereby, pharmacological disturbance of the MDM2-p53 interaction might lead to p53 stabilization and increased transcriptional activity independent of irradiation. Treatment of D341 and HCT116 cells with MI-773 induced PlGF-expression at an early time point and resulted in enhanced PlGF levels in the supernatant as determined 24 hours after MI-773 treatment start (Figure 3A-D).

Putative regulation of PlGF by p53 was more specifically investigated using p53directed siRNA. p53 mRNA and protein levels were downregulated on siRNAtreatment in both D341 and HCT116 cells for up to 96 hours. Likewise, expression of the well-known p53 downstream target p21 was also downregulated (Supplementary figure 3A-C). Tumor cells were irradiated 48 hours after siRNA transfection and PlGF secretion was determined in the CM 48 hours after irradiation. PlGF levels were strongly enhanced in response to irradiation in siLuc-pretreated cells, but basal and irradiation-induced PlGF-levels were significantly decreased in CM of sip53-pretreated cells (Figure 3E-F).

MDM2 has additional binding targets besides p53 and thereby MI-773 might reactivate also other transcriptional activities besides p53 leading to enhanced PlGF-expression. Therefore, PlGF-expression and secretion was also analyzed in siRNA-p53downregulated cells on treatment with MI-773 for 4 and 24 hours. Neither expression nor secretion was enhanced by MI-773 in p53-downregulated cells. (Figure 4A-D).

Additional experiments were performed with the p53-inhibitor Pifithrin (PFT), which prevents nuclear translocation of p53 and transcriptional activation of downstream genes [41, 42]. Prior to irradiation, cells were preincubated with Pifithrin for 2 hours and the PlGF level determined 24 hours thereafter. Similar to the complementary experiments performed with the MDM2-inhibitor and p53-directed siRNA, decreased PlGF levels in Pifithrin-pretreated cells indicate p53-mediated PlGF regulation in response to irradiation (Figure 4E-F).

Overall, these complementary experiments strongly suggest that p53 is a major regulator for PlGF expression.

To determine whether p53 truly binds to promoter region of PlGF, we performed additional ChIP-qPCR assay. Unfortunately, we could not identify p53 binding site on PlGF promoter using p53-antibodies and PlGF site-specific primers mentioned by Rashi-Elkeles et al. [38]. Binding of p53 to a different enhancer region in PlGF promoter is possible in another cell type.

The effect of BAY 87-2243 on PlGF secretion under hypoxia

Contradictory results exist on the expression of PlGF under hypoxic stress conditions, which might be cell type and genetic background dependent [35-37]. To investigate IR-induced PlGF-secretion under hypoxic conditions, D341, p53-wildtype, and DAOY, p53-mutated medulloblastoma cells, were irradiated followed by incubation under normoxic and hypoxic (1% O_2) conditions. In addition, cells were pre-incubated with increasing concentrations of BAY 87-2243 (0nM, 10nM, 50nM) one hour prior to IR. BAY 87-2243 is a potent inhibitor for hypoxia-induced, HIF-1α and HIF-2α-mediated gene activation [43]. Due to delayed expression of PlGF in cells with mutated p53, secreted PlGF was determined in CM only 48 hours after irradiation. Hypoxia minimally enhanced basal and IR-induced PlGF secretion in the p53-wildtype medulloblastoma cells and cellular pretreatment with BAY 87-2243 did not interfere with PlGF secretion neither under normoxic nor hypoxic conditions. At the same time, cellular pretreatment with BAY 87-2243 abrogated IR-induced PlGF-secretion in the p53 mutated DAOY cells under hypoxic conditions (Figure 5A-B). Detection of HIF1α in non-irradiated and irradiated DAOY cells and incubated under hypoxic conditions also demonstrated strongly reduced HIF-1α-levels in cells pretreated with BAY 872243 (Figure 5C).

Control experiments were also performed with D431 cells transfected with p53oriented siRNA and incubated under hypoxic conditions (1% O_2). Hypoxia alone did not affect PlGF secretion while irradiation resulted in partially enhanced PlGF secretion from cells incubated under hypoxic conditions. Similar to the experiments performed under normoxic condition, downregulation of p53 by p53-directed siRNA strongly abolished irradiation-induced PlGF secretion also under hypoxic condition (Figure 5D).

These results strongly suggest differential hypoxia-dependent regulation of PlGFsecretion in p53-wildtype and p53-mutated medulloblastoma cells. While

PlGFsecretion is predominantly p53-regulated but in a HIF-signaling-independent way in p53-wildtype cells, interference with HIF-signaling by BAY 87-2243 downregulates PlGF-secretion in cells with a p53-mutated background.

Angiogenesis is inhibited in PlGF-ko cells

To further investigate auto- and paracrine effects of IR-induced secretion of PlGF, D341 PlGF knockout cells were generated using two different exon 3-targeting single guide RNAs of the PlGF gene and cloned into a GFP and Cas9 expressing pLentiCRISPR plasmid. Sequencing of the targeting region of exon 3 in our cell clones confirmed successful PlGF-gene targeting (Supplementary figure 3A). IR-induced PlGF secretion was determined in the cell supernatants derived from a control cell clone and a selected knock out cell clone 24 hours and 48 hours after irradiation. PlGF was undetectable in the supernatants of PlGF knockout cell line and IR-enhanced PlGF-levels were determined in the supernatants of the control cell line (PlGF-wt) (Supplementary figure 3B). In order to investigate a paracrine effect of PlGF, we performed a Boyden chamber migration assay with irradiated human umbilical vein endothelial cells (HUVECs) seeded in the upper chamber migrating towards nonirradiated and irradiated D341 PlGF-wt and D341 PlGF-ko cells, respectively. No quantitative difference in HUVEC migration towards unirradiated PlGF-wt versus unirradiated PlGF-ko cells could be determined. However, significantly increased migration of HUVECs towards irradiated PlGF-wt cells versus irradiated PlGF-ko cell could be observed (Figure 6A).

Prior to in vivo experiments, proliferative activity and clonogenicity of PlGF-wt and PlGF-ko cell clones were determined and revealed only minor and PlGF-independent differential readouts (Supplementary figure 3C-D).

To determine a role of IR-regulated PlGF-expression on the endothelial compartment in vivo, medulloblastoma xenografts were developed from subcutaneously-injected PlGF-wt and PlGF-ko tumor cells. Overall, 83-87% of mice injected with PlGF-wt cells and 52-58% of mice injected with PlGF-ko cells developed tumors. Days to reach a treatment volume of 150-200mm^3 ranged from 31-37 days for PlGF-wt and 36-50 days for PlGF-ko cells respectively. Tumors were irradiated with increasing doses of IR (0Gy, 5Gy, 10Gy) and mice were sacrificed eight days following irradiation. Tumor sections were stained for H&E, CD31 and SMA and the amount of vessels were

counted in at least ten fields for each tumor. Microvessel densities (MVD) (CD31staining) in PlGF-wt and PlGF-ko medulloblastoma xenografts were comparable, and MVD only minimally changed in PlGF-wt tumor xenografts in response to irradiation. Interestingly though, a decrease in MVD was observed in tumor xenografts derived from PlGF-ko-medulloblastoma cells irradiated with 5Gy and MVD was strongly reduced on irradiation with 10Gy (Figure 6B). A quantitative similar outcome was determined on smooth muscle actin (SMA) staining as an indicator of pericyte coverage and vessel functionality (Figure 6C). Decreased pericyte coverage indicated increased radiosensitivity in PlGF-ko tumors. Representative stainings of tumor sections are shown in Figure 6D.

PlGF-wt and PlGF-ko cells displayed comparable proliferative activity and radiosensitivity in vitro (see above). However, the determination of a short-time efficacy-oriented endpoint in vivo revealed enhanced radiosensitivity of PlGF-ko tumors in comparison PlGF-wt tumors. In response to 5 and 10Gy, 3 out of 5 PlGF-ko tumors (60%) in each group showed complete tumor regression in comparison to only 1 PlGF-wt tumor in the group of mice treated with 10Gy of IR (Figure 7).

Overall, these results suggest that tumor-derived PlGF has a tumor vasculature protective effect and thereby co-determines tumor radiosensitivity in vivo.

Discussion

Anti-angiogenic agents are mainly directed against the VEGF/VEGFR signaling pathway. These agents present prolonged survival, but do not cure cancer. Targeting the VEGF/VEGFR pathway eventually leads to resistance and to an angiogenic switch to other growth factors, such as PlGF, independent of VEGF [29]. The expression level of placental growth factor is increased in several cancer types and correlates with poor survival, cancer progression and resistance to therapy [19-22]. Patients with metastatic colorectal cancer have shown increased PlGF levels after combined treatment with bevacizumab, chemotherapy and radiation [44, 45]. Furthermore, increased PlGF expression has been linked to increased vessel number, size and permeability [24] and targeting of PlGF demonstrated reduced tumor vascularization in VEGFR-inhibitor resistant tumor model [29]. However, clinical studies with PlGF neutralizing antibodies did not show improved survival in combination with

bevacizumab in previously treated glioblastoma patients and therefore the strategy to target PlGF was discontinued, despite minimal normal tissue toxicities [46-48]. Nevertheless, and taking its undisputed but still not fully understood role in pathological angiogenesis into consideration, PlGF could still represent an interesting target for cancer treatment. However, detailed mechanistic investigations on its regulation alone and as part of a combined treatment modality are required.

Here, we investigated PlGF expression and secretion in multiple cancer cell lines with differential genetic backgrounds in response to increasing doses of IR. Treatmentinduced PlGF expression could be detected in some cell lines already at early time point after irradiation, whereas other cell lines only responded in a delayed way or did not show any increase of IR-induced PlGF-expression at all. Based on a literature search, this differential response could be correlated with the mutational status of the tumor suppressor p53. At the same time, we proposed that other transcription factors, such as HIFs, could play a role in PlGF regulation in cells with mutated p53 and relevant for delayed expression in response to irradiation.

The PlGF promoter contains binding sites for multiple transcription factors including p53. It has four NF-kB, five metal transcription factor 1 (MTF1), three Sp1, 1 BF2 binding sites and a predicted hypoxia responsive element (HRE) in its promoter. However, all the binding sites were identified in different cell types (HEK, immortalized mouse embryonic fibroblasts, HeLa cells) and under different conditions [37, 49, 50]. First indications that p53 might regulate PlGF-expression derive from a wide-scale transcriptome study in normal human B-lymphoblastoid cells (TK6 cells) investigating the transcriptional activity of p53 in response to ionizing radiation. Binding of p53 to the PlGF-promoter was also confirmed by ChIP assay but has not been further investigated beyond these transcriptional studies [38]. Despite several attempts we could not perform successful ChIP assays demonstrating direct binding of p53 to the promoter site of PlGF in the medulloblastoma. This might be due to technical limitations or the intrinsic challenge to detect direct promoter binding of p53 due to the multiple layers of different transcriptional regulation of p53 downstream targets in response to stress [51]. Nevertheless, our expression and secretion studies performed in multiple cell lines clearly demonstrate that p53 plays a major role in PlGF regulation in response to ionizing radiation.

In order to support our hypothesis regarding PlGF regulation by p53, we first demonstrated that PlGF is upregulated in response to p53 activation by other means than IR. Cellular treatment of p53-wt tumor cells with the MDM2 inhibitor MI-773 stabilized p53 and subsequently resulted in increased PlGF expression and secretion with similar kinetics to IR-induced PlGF-expression and secretion in these p53-wt tumor cells. Downregulation of p53 transcriptional activity using either p53-directed siRNA or the small molecular p53-inhibitory compound Pifithrin corroborated p53mediated PlGF-expression in response to irradiation. The PlGF promoter has additional binding sites for other transcription factors than p53, such as NF-kB as mentioned above, that could also potentially be affected by MDM2. Control experiments with the MDM2-inhibitor with concomitant downregulation of p53, indicated that p53 is the major transcription factor relevant for IR-mediated regulation of PlGF in p53-wildtype tumor cells.

We hypothesized that other transcription factors, mainly HIFs, could be the regulators of PlGF-expression in p53 mutated cells. In order to support this hypothesis, we investigated PlGF secretion in BAY 87-2243-treated p53 wildtype and p53 mutated tumor cells both under normoxic and hypoxic conditions. While the HIF-inhibiting compound BAY 87-2243 did not affect PlGF under either conditions in p53-wildtype cells, BAY 87-2243 downregulated hypoxia-induced and IR-induced PlGF-secretion under hypoxic conditions in a p53-mutated background. Our results support investigations performed by others showing increased PlGF expression under hypoxic condition. Though, none of these studies demonstrate hypoxia responsive elements in the PlGF promoter region [49, 50, 52]. At the same time, we detect for the first time differential PlGF-expression in dependence on differential genetic backgrounds and environmental conditions, in particular p53 and hypoxia, in response to irradiation. In depth mechanistic investigations are now required to dissect potential competitive elements for PlGF regulation. Furthermore, BAY 87-2243 inhibits HIF indirectly through inhibition of mitochondrial complex I and decreased ROS levels [53]. Due to limited information about BAY 87-2243, we cannot exclude at this stage that additional HIF-independent mechanisms contribute to the regulation of PlGF-expression under hypoxic conditions in response to IR and independent of p53.

In order to study the role of PlGF both in vitro and in vivo, D341-based PlGF knockout cell clones were generated, with undetectable PlGF secretion in conditioned media

thereof. As a functional paracrine endpoint for PlGF migration of endothelial cells was investigated. While no difference in endothelial cell migration towards non-irradiated PlGF-wt and PlGF-ko tumor cells, respectively, could be detected, increased endothelial cell migration was only observed towards irradiated, PlGF-secreting cells. These results indicated a specific paracrine (pro-)angiogenic role for PlGF under stress but not under basal condition.

PlGF is known to be involved in angiogenesis [16]. Based on these IR-induced PlGF mediated effect in vitro, we therefore aimed to investigate its IR-induced role in angiogenesis and response to irradiation in medulloblastoma xenografts derived from PlGF-wt and PlGF-ko-cells. While tumor growth rate and basal tumor angiogenesis was only minimally affected by the PlGF-status, the microvessel density was strongly reduced in irradiation PlGF-ko tumors. Enhanced vascular radiosensitivity correlated with increased radiosensitivity of PlGF-ko-tumors leading to a high level of tumor regression in these tumors in comparison to PlGF-wildtype tumors.

Overall, these results indicate that PlGF plays a minor role for the formation of an intact vasculature during tumor growth. On the other hand, PlGF has a tumor vasculature protective role in response to irradiation, which might be mediated by IR-induced secretion of PlGF from the irradiated tumor cells. Lack of this protective role could contribute to a microenvironment-mediated increase of tumor radiosensitivity. Thus, such a role for PlGF represents an interesting rationale for a combined treatment modality with ionizing radiation and/or chemotherapy.

Acknowledgements

This work was supported by grants from European Union's Horizon 2020 research and innovation program (Marie Sklodowska-Curie ITN RADIATE, 642623) and Hartmann Müller-Stiftung (2333). Authors thank Department of Pathology, University Hospital Zurich, Institute of Anatomy and Center for Microscopy and Image Analysis, University of Zurich for their help with tumor sections. We also thank Beat Schäfer and Martin Baumgartner for kindly providing pLentiCRISPR plasmid and medulloblastoma cell lines, respectively.

Figure legends

Figure 1. PlGF secretion is increased in multiple cell lines after irradiation. A) Bioplex-based quantification of 32 cancer biomarkers in the supernatant from irradiated A549 lung adenocarcinoma cells with strongly increased PlGF levels determined 24 hours after irradiation. **B – F)** Irradiation-induced PlGF-expression was investigated across multiple tumor cell lines derived from colon (HCT116), medulloblastoma (D341 and DAOY) and lung carcinoma (A549 and H125). Supernatants were analyzed for PlGF secretion 24 and 48 hours after irradiation. IRenhanced PlGF-secretion was identified in the conditioned media (CM) from all cell lines by ELISA in a time- and IR-dose-dependent manner. Level of PlGF secretion differed between different cell lines and therefore graphs show adjusted y-axis for each cell line. Bar graphs represent concentration of PlGF normalized to viable cell count ± SEM. Students t-test was used for statistical analysis.

Figure 2. Relative PlGF mRNA expression is increased in response to IR. qRTPCR-based relative PlGF mRNA expression determined in colon (HCT116), medulloblastoma (D341 and DAOY) and lung carcinoma (A549 and H125) cells 4 hours **(A – E)** and 24 hours (**F – J)** after irradiation with increasing doses of ionizing radiation. Early and delayed upregulation of PlGF mRNA in response to irradiation correlated with early and delayed PlGF secretion, respectively (Fig. 1). Bar graphs show PlGF mRNA expression relative to GAPDH ± SEM. Students t-test was used for statistical analysis.

Figure 3. p53 is a major regulator of PlGF-expression in response to IR. Treatment of D341 **(A – B)** and HCT116 **(C – D)** cells with increasing doses of the MDM2-inhibitor MI-773 induced PlGF-expression at 4 hours and resulted in enhanced PlGF levels in the supernatant as determined 24 hours after MI-773 treatment start. **E – F)** Downregulation of p53 with p53-directed siRNA abolished IR-induced PlGFsecretion. Cells were irradiated 48 hours after downregulation of p53 by siRNA targeting. PlGF secretion was determined in the CM 48 hours after irradiation. siLuc treated cells demonstrated a strong increase in PlGF secretion upon irradiation. Bar graphs show PlGF mRNA expression relative to GAPDH and PlGF secretion ± SEM. Students t-test was used for statistical analysis.

Figure 4. The MDMD2-p53 axis specifically regulates PlGF expression A – D) PlGF expression and secretion in p53-downregulated D341 **(A – B)** and HCT116 **(C – D)** cells on treatment with increasing doses of MI-773 for 4 and 24 hours to investigate if other MDM2 targets besides p53 might be involved in PlGF regulation. **E – F)** 2 hours prior to irradiation, cells were treated with the p53-inhibitor Pifithrin (PFT). PlGF secretion was determined 24 hours thereafter in the CM. IR-induced PlGF secretion from D341 and HCT116 cells was inhibited by PFT. Bar graphs show PlGF mRNA expression relative to GAPDH and PlGF secretion ± SEM. Students t-test was used for statistical analysis.

Figure 5. BAY 87-2243 inhibits PlGF secretion in p53-mutated but not in p53-wt tumor cells under hypoxic conditions. A – B) p53 wildtype D341 and p53 mutated DAOY medulloblastoma cells were pre-treated with increasing doses of BAY 87-2243 one hour prior to IR. Following irradiation, cells were incubated under normoxic and hypoxic (1% O_2) conditions for 48 hours and PlGF was determined in the CM by ELISA. **C)** HIF-1α protein level in BAY 87-2243 pretreated, non-irradiated and irradiated DAOY cells under normoxic and hypoxic conditions as determined in cellular lysates by western blotting. **D)** PlGF-secretion in siLuc- and sip53-targeted D341 cells incubated under hypoxic conditions (1% O_2) for 48 hours following irradiation. Downregulation of p53 by p53-directed siRNA abolished irradiation-induced PlGF secretion under hypoxic condition. Graphs show secreted PlGF levels ± SEM. Students t-test was used for statistical analysis.

Figure 6. PlGF protects the tumor vasculature in response to irradiation. A) The paracrine effect of PlGF on HUVECs was investigated by a Boyden chamber migration assay. HUVECs were seeded in the upper chamber and irradiated, and the amount of migrated HUVECs towards non-irradiated and irradiated D341 PlGF-wt and D341 PlGF-ko cells, respectively, was determined by DAPI staining. **B – C)** Microvessel densities were determined in tissue sections from tumor xenografts derived from PlGFwt and PlGF-ko D341 medulloblastoma cells. Tumors were harvested 8 days following irradiation and tissue sections were stained for CD31- and SMA. The amount of vessels was counted in at least ten fields for each tumor. Each dot shows amount of vessels in one field. **D)** Representative tissue sections for each treatment group. Scale bar shows 50µm. One-way ANOVA and Students t-test was used for statistical analysis.

Figure 7. PlGF determines medulloblastoma radiosensitivity. Tumor xenografts derived from PlGF-wt and PlGF-ko D341 medulloblastoma cells were sham-irradiated or irradiated with 5 and 10 Gy when tumors reached a volume between 150mm^3 and 200mm^3. The responses of the individual tumors are depicted. In response to 5 and 10Gy, 3 out of 5 PlGF-ko tumors of each irradiated group (60%) showed complete tumor regression in comparison to only 1 PlGF-wt tumor in the group of mice treated with 10 Gy of IR.

Supplementary figure legends

Supplementary figure 1. Relative PlGF mRNA expression is increased in response to IR. A – D) qRT-PCR-based relative PlGF mRNA expression was determined in lung adenocarcinoma (H292 and H460) cells 4 hours and 24 hours after irradiation with increasing doses of ionizing radiation. **E – J)** Minimal or no change in relative PlGF mRNA expression in lung adenocarcinoma (H358), head and neck squamous carcinoma (FaDu) and medulloblastoma (UW228) cells after irradiation.

Bar graphs show PlGF mRNA expression relative to GAPDH ± SEM. Students t-test was used for statistical analysis.

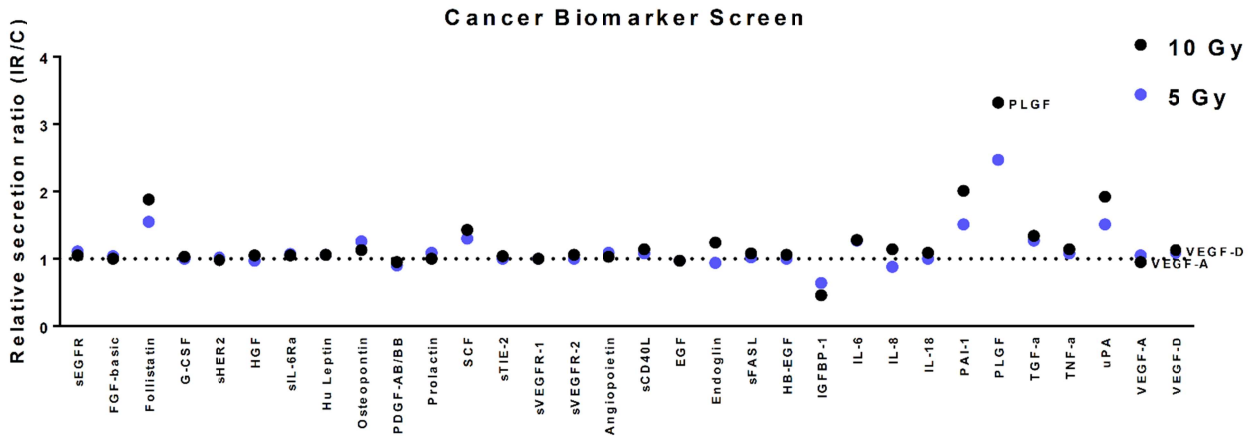
Supplementary table 1. Correlation of genetic background of cell lines to early PlGF expression in response to IR.

Supplementary figure 2. p53 is a major regulator of PlGF in response to IR. Downregulation of p53 with p53-directed siRNA in D341 **(A)** and HCT116 **(B)** cells. Relative p53 mRNA expression was determined 48-96 hours after p53 downregulation. **C)** p53 protein level determined in cell lysates of D341 cells 48-96 hours after p53 downregulation. As a control and downstream target of p53, p21 levels are represented following sip53 treatment. Graphs show p53 mRNA expression relative to GAPDH ± SEM.

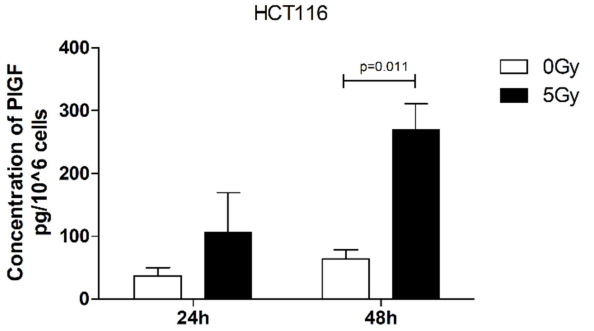
Supplementary figure 3. Generation of PlGF knockout cell lines using CRISPR/Cas9 system. A) A non-targeting (Ct) and an exon 3-targeting single guide RNA of the PlGF gene were separately cloned into the Cas9 and EGFP expressing pLentiCRISPR plasmids. Sequencing of the targeted region of exon 3 revealed successful PlGF-gene targeting. Aligned sequences of exon 3 of wildtype (upper sequence) and transfected cell lines (lower sequence) was compared. Successful knockout is shown by an insertion of a nucleotide in the knockout cell line (red square). **B)** IR-induced PlGF secretion was determined in the supernatants of the cell clones 24 hours and 48 hours after irradiation. **C)** Clonogenicity and **D)** proliferative activity of PlGF-wt and PlGF-ko cell clones in response to irradiation. Experiments were performed once and twice, respectively.

Figure 1

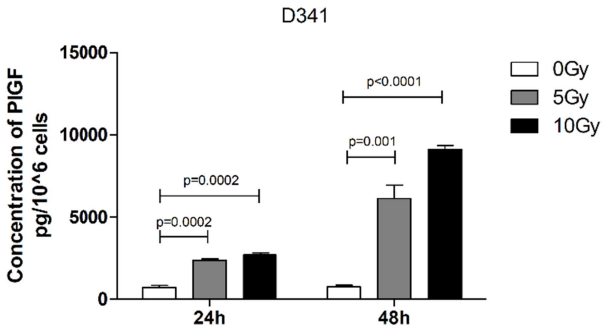
A)


Cancer Biomarker Screen
Relative secretion ratio (IR/C)
10 Gy
5 Gy
PLGF
VEGF-D
VEGF-A
sEGFR
FGF-basic
Follistatin
G-CSF
sHER2
HGF
sIL-6Ra
Hu Leptin
Osteopontin
PDGF-AB/BB
Prolactin
SCF
sTIE-2
sVEGFR-1
sVEGFR-2
Angiopoietin
sCD40L
EGF
Endoglin
sFASL
HB-EGF
IGFBP-1
IL-6
IL-8
IL-18
PAI-1
PLGF
TGF-a
TNF-a
uPA
VEGF-A
VEGF-D

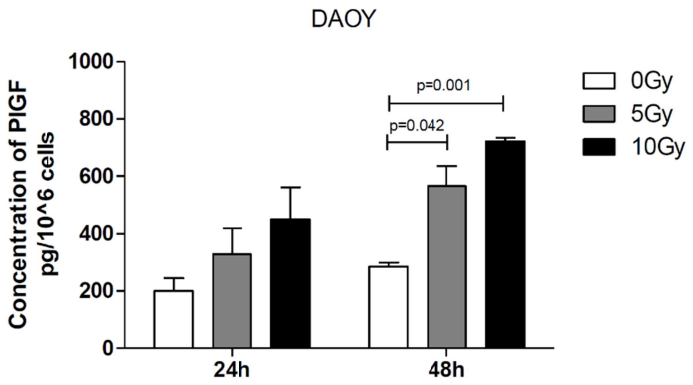
B)


HCT116
Concentration of PlGF pg/10^6 cells
p=0.011
0Gy
5Gy
24h
48h

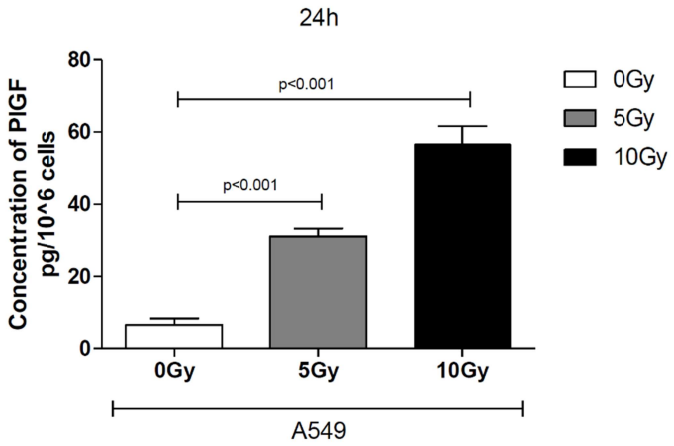
C)


D341
Concentration of PlGF pg/10^6 cells
p=0.0002
p=0.0002
p<0.0001
p=0.001
0Gy
5Gy
10Gy
24h
48h

D)


DAOY
Concentration of PlGF
pg/10^6 cells
1000
800
600
400
200
0
p=0.001
p=0.042
0Gy
5Gy
10Gy
24h
48h

E)


24h
Concentration of PlGF
pg/10^6 cells
80
60
40
20
0
p<0.001
p<0.001
0Gy
5Gy
10Gy
0Gy
5Gy
10Gy
A549

F)

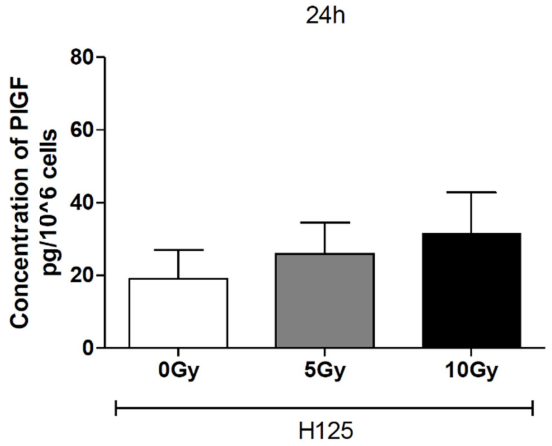
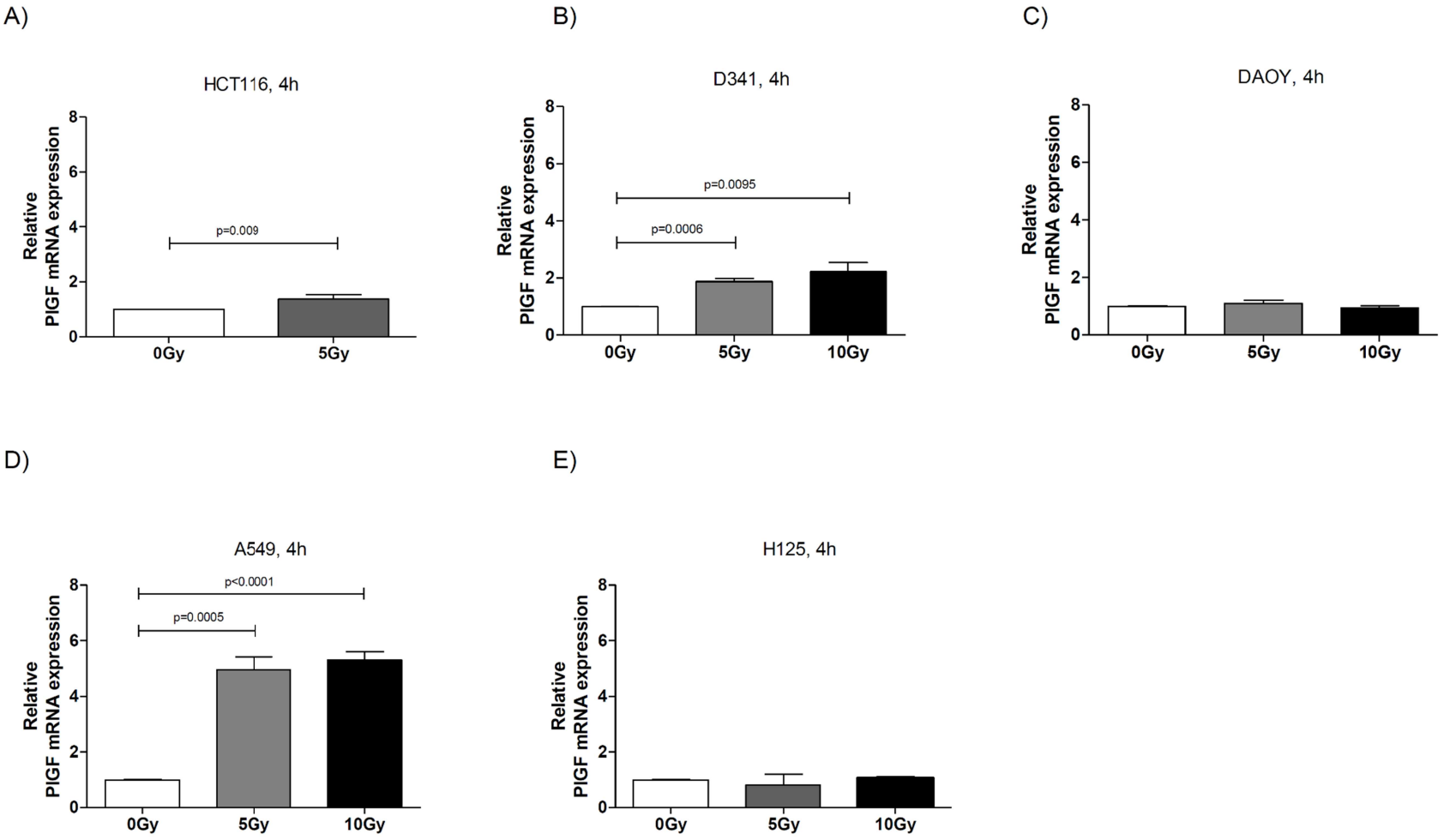
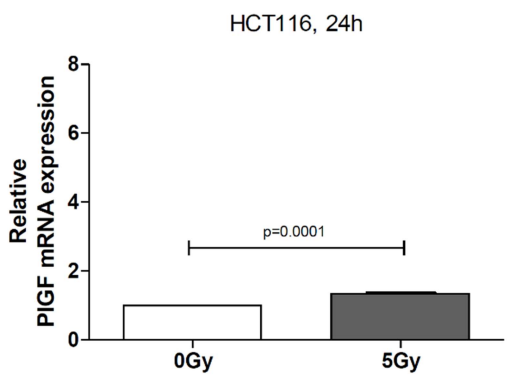

24h
Concentration of PlGF
pg/10^6 cells
80
60
40
20
0
0Gy
5Gy
10Gy
H125

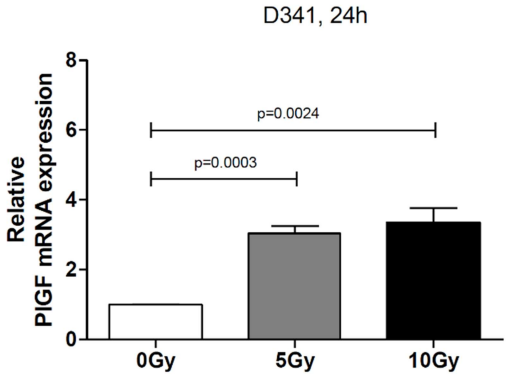
Figure 2


A)
HCT116, 4h
Relative PIGF mRNA expression
p=0.009
0Gy
5Gy
B)
D341, 4h
Relative PIGF mRNA expression
p=0.0095
p=0.0006
0Gy
5Gy
10Gy
C)
DAOY, 4h
Relative PIGF mRNA expression
0Gy
5Gy
10Gy
D)
A549, 4h
Relative PIGF mRNA expression
p<0.0001
p=0.0005
0Gy
5Gy
10Gy
E)
H125, 4h
Relative PIGF mRNA expression
0Gy
5Gy
10Gy

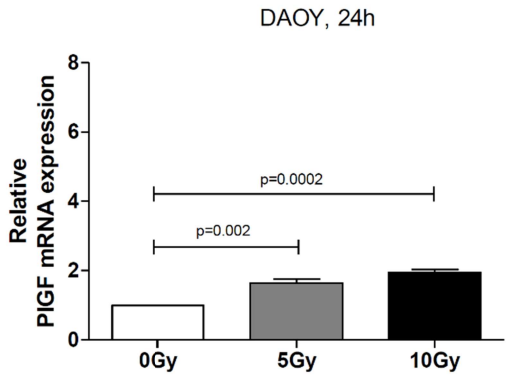
F)


HCT116, 24h
Relative PIGF mRNA expression
0
2
4
6
8
p=0.0001
0Gy
5Gy

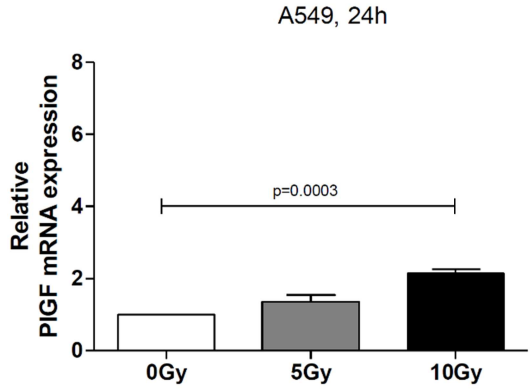
G)


D341, 24h
Relative PIGF mRNA expression
0
2
4
6
8
p=0.0024
p=0.0003
0Gy
5Gy
10Gy

H)


DAOY, 24h
Relative PIGF mRNA expression
0
2
4
6
8
p=0.0002
p=0.002
0Gy
5Gy
10Gy

I)


A549, 24h
Relative PIGF mRNA expression
0
2
4
6
8
p=0.0003
0Gy
5Gy
10Gy

J)

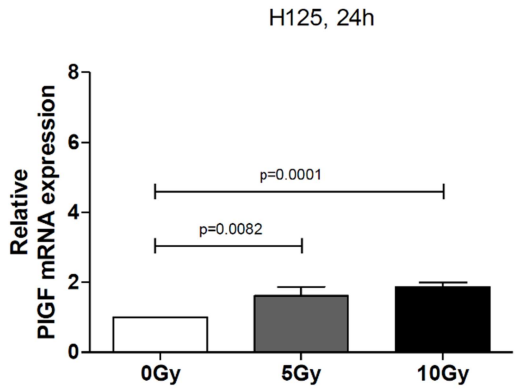
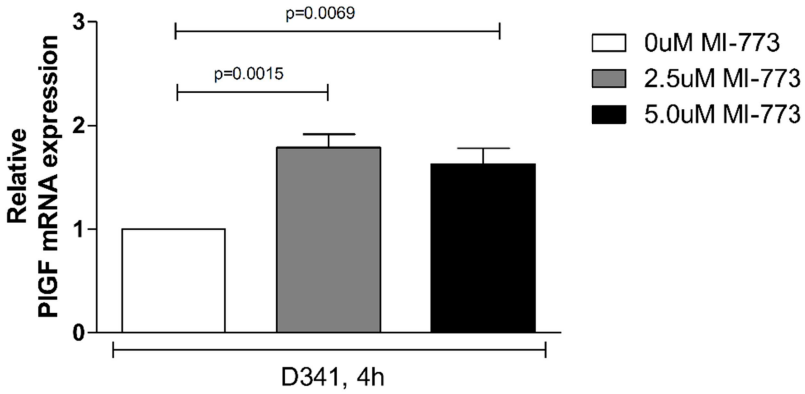
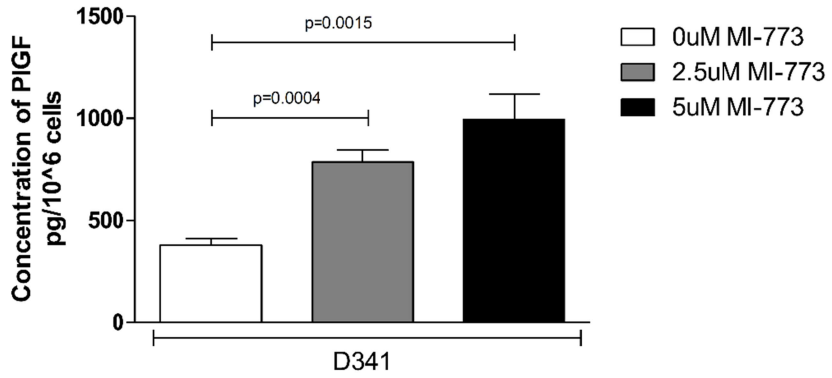

H125, 24h
Relative PIGF mRNA expression
0
2
4
6
8
p=0.0001
p=0.0082
0Gy
5Gy
10Gy

Figure 3

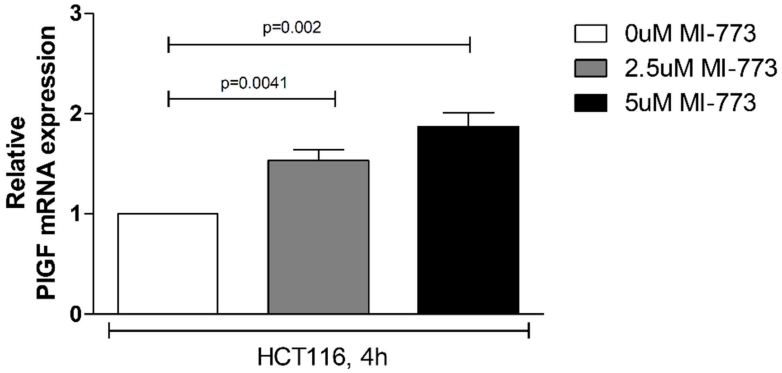
A)



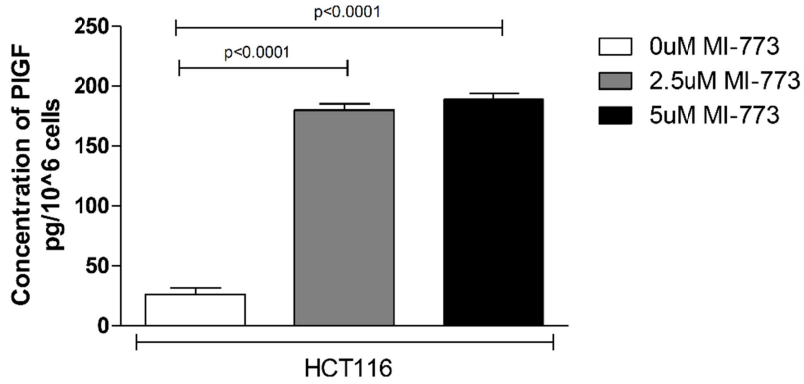
B)



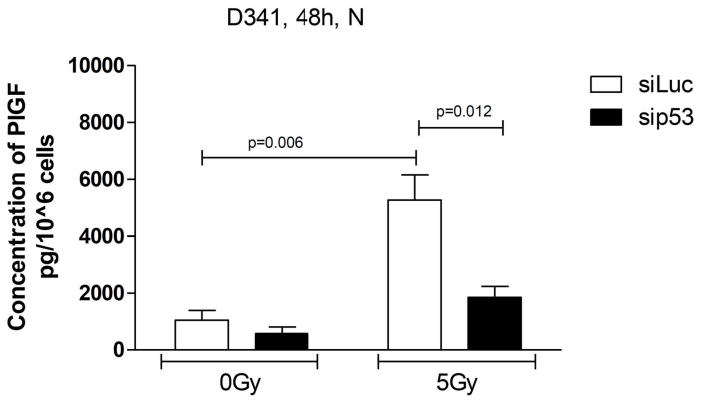
C)



D)



E)


D341, 48h, N
Concentration of PIGF
pg/10^6 cells
10000
8000
6000
4000
2000
0
p=0.006
p=0.012
0Gy
5Gy
siLuc
sip53

F)

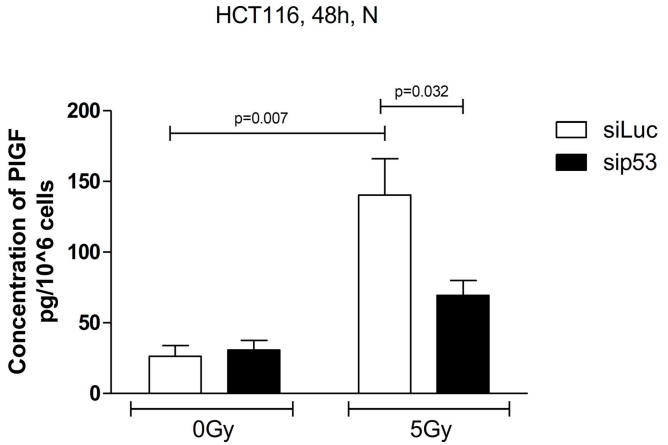
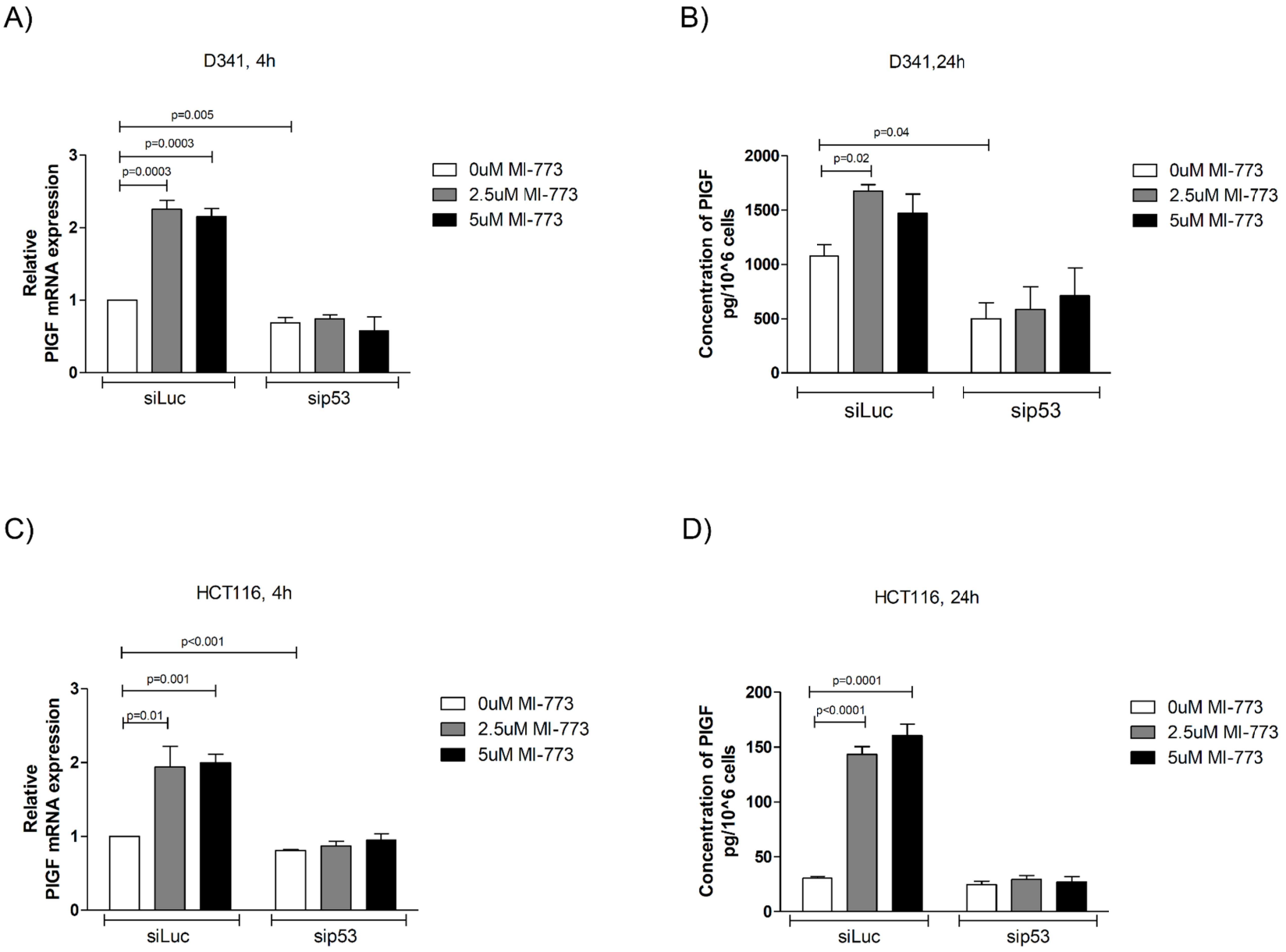
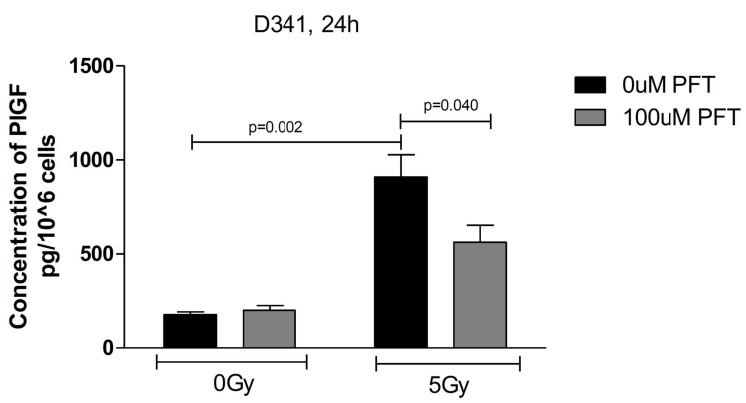

HCT116, 48h, N
Concentration of PIGF
pg/10^6 cells
200
150
100
50
0
p=0.007
p=0.032
0Gy
5Gy
siLuc
sip53

Figure 4


A)
D341, 4h
p=0.005
p=0.0003
p=0.0003
Relative PIGF mRNA expression
0
1
2
3
siLuc
sip53
0uM MI-773
2.5uM MI-773
5uM MI-773
B)
D341,24h
p=0.04
p=0.02
Concentration of PIGF pg/10^6 cells
0
500
1000
1500
2000
siLuc
sip53
0uM MI-773
2.5uM MI-773
5uM MI-773
C)
HCT116, 4h
p<0.001
p=0.001
p=0.01
Relative PIGF mRNA expression
0
1
2
3
siLuc
sip53
0uM MI-773
2.5uM MI-773
5uM MI-773
D)
HCT116, 24h
p=0.0001
p<0.0001
Concentration of PIGF pg/10^6 cells
0
50
100
150
200
siLuc
sip53
0uM MI-773
2.5uM MI-773
5uM MI-773

E)


D341, 24h
Concentration of PIGF
pg/10^6 cells
1500
1000
500
0
p=0.002
p=0.040
0uM PFT
100uM PFT
0Gy
5Gy

F)

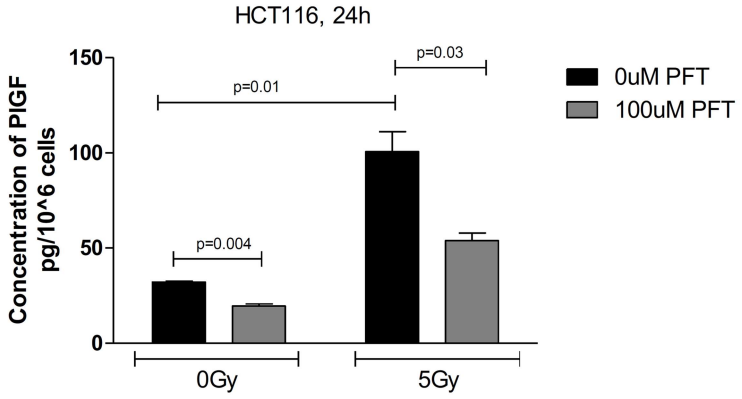
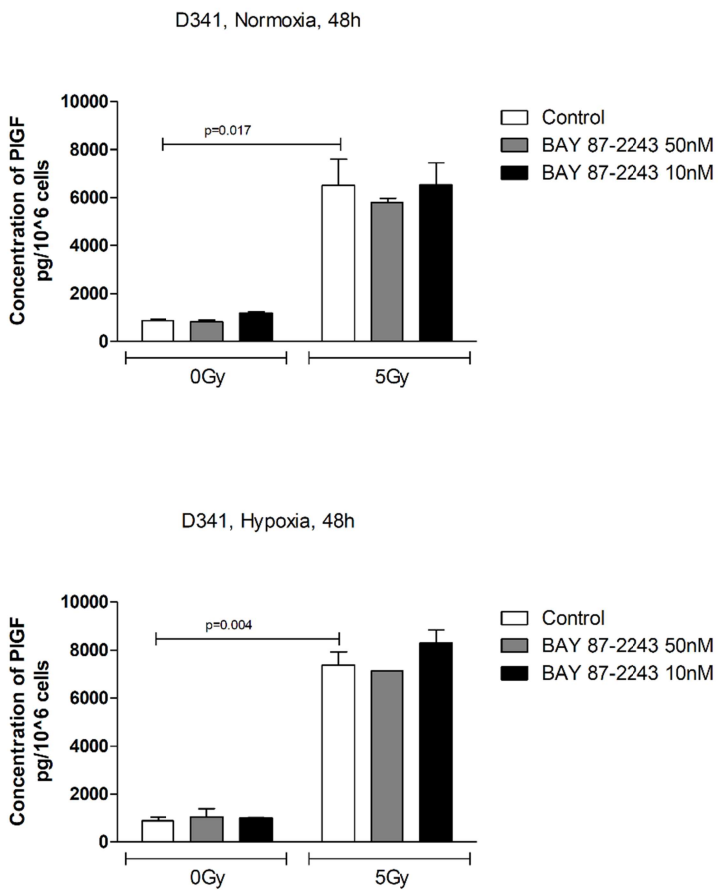
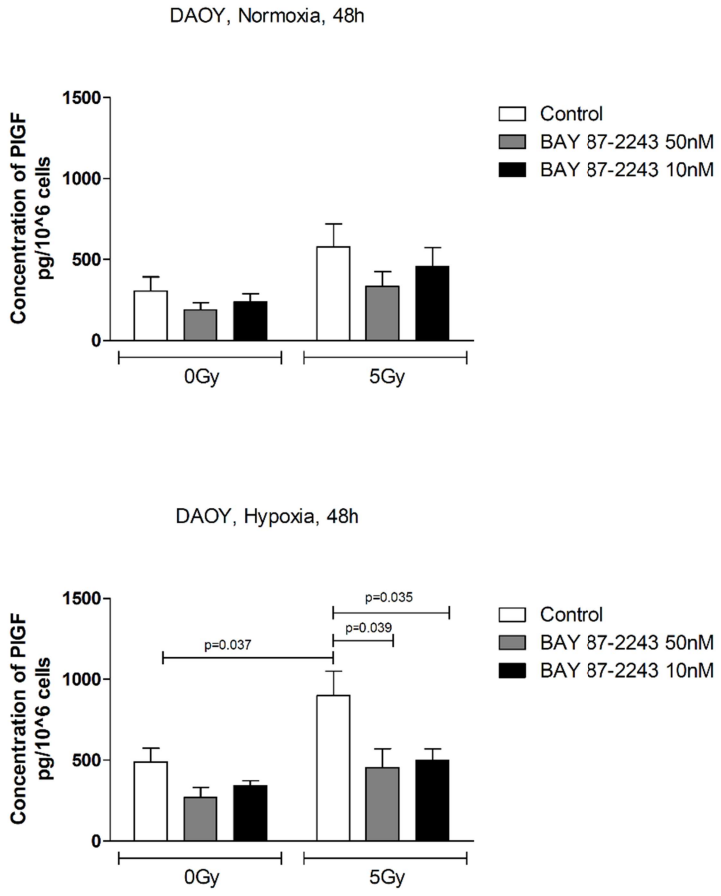

HCT116, 24h
Concentration of PIGF
pg/10^6 cells
150
100
50
0
p=0.01
p=0.03
p=0.004
0uM PFT
100uM PFT
0Gy
5Gy

Figure 5

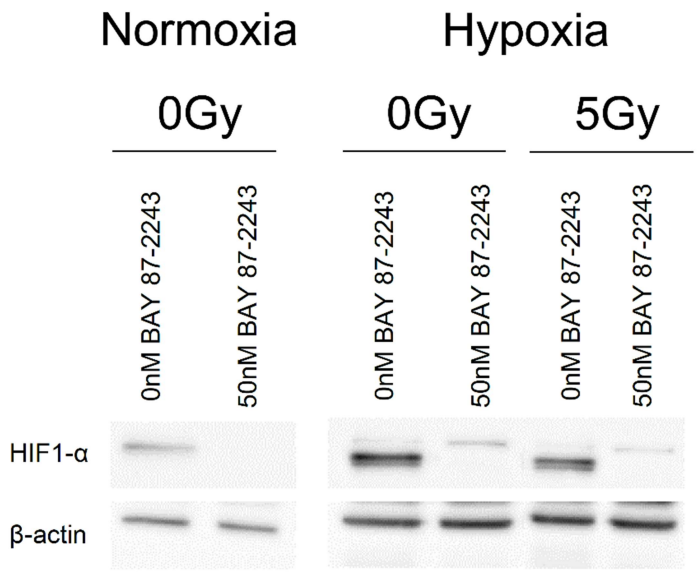
A)


D341, Normoxia, 48h
Concentration of PIGF pg/10^6 cells
10000
8000
6000
4000
2000
0
p=0.017
0Gy
5Gy
Control
BAY 87-2243 50nM
BAY 87-2243 10nM
D341, Hypoxia, 48h
Concentration of PIGF pg/10^6 cells
10000
8000
6000
4000
2000
0
p=0.004
0Gy
5Gy
Control
BAY 87-2243 50nM
BAY 87-2243 10nM

B)


DAOY, Normoxia, 48h
Concentration of PIGF pg/10^6 cells
1500
1000
500
0
0Gy
5Gy
Control
BAY 87-2243 50nM
BAY 87-2243 10nM
DAOY, Hypoxia, 48h
Concentration of PIGF pg/10^6 cells
1500
1000
500
0
p=0.037
p=0.039
p=0.035
0Gy
5Gy
Control
BAY 87-2243 50nM
BAY 87-2243 10nM

C)


Normoxia
Hypoxia
0Gy
0Gy
5Gy
0nM BAY 87-2243
50nM BAY 87-2243
0nM BAY 87-2243
50nM BAY 87-2243
0nM BAY 87-2243
50nM BAY 87-2243
HIF1-α
β-actin

D)

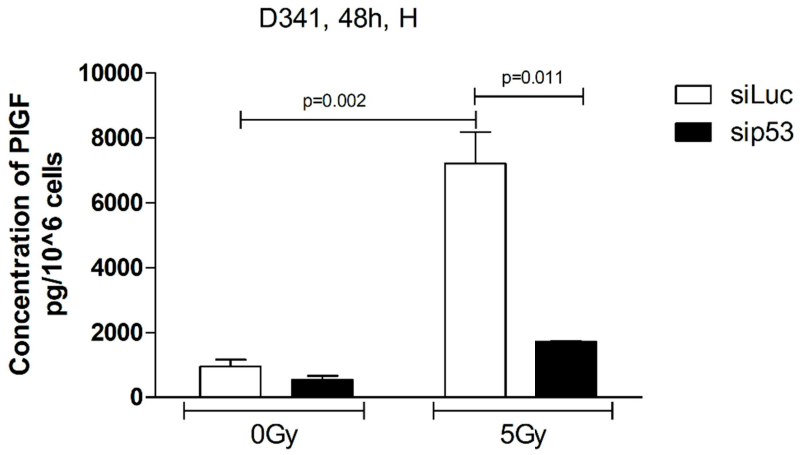
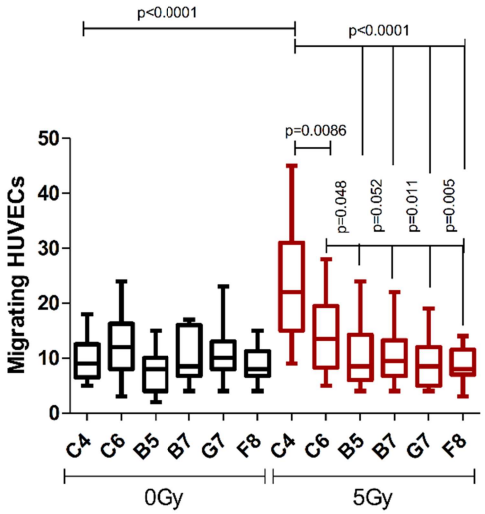
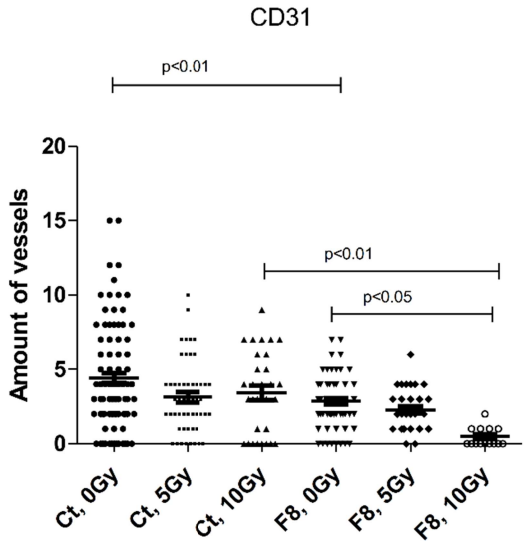

D341, 48h, H
Concentration of PIGF pg/10^6 cells
10000
8000
6000
4000
2000
0
p=0.002
p=0.011
siLuc
sip53
0Gy
5Gy

Figure 6

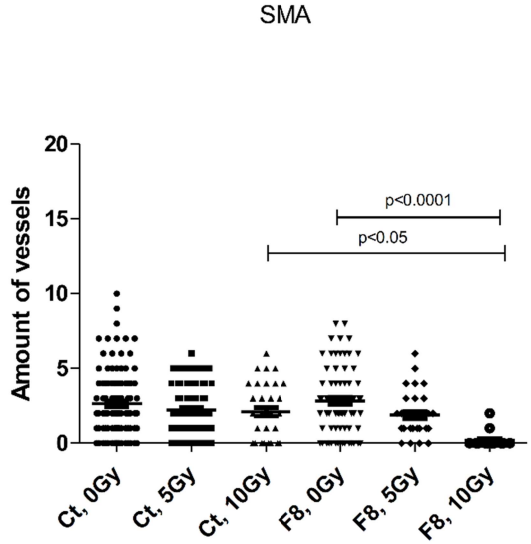
A)


p<0.0001
p<0.0001
p=0.0086
p=0.048
p=0.052
p=0.011
p=0.005
Migrating HUVECs
50
40
30
20
10
0
C4
C6
B5
B7
G7
F8
C4
C6
B5
B7
G7
F8
0Gy
5Gy

B)


CD31
p<0.01
p<0.01
p<0.05
Amount of vessels
20
15
10
5
0
Ct, 0Gy
Ct, 5Gy
Ct, 10Gy
F8, 0Gy
F8, 5Gy
F8, 10Gy

C)


SMA
p<0.0001
p<0.05
Amount of vessels
20
15
10
5
0
Ct, 0Gy
Ct, 5Gy
Ct, 10Gy
F8, 0Gy
F8, 5Gy
F8, 10Gy

D)

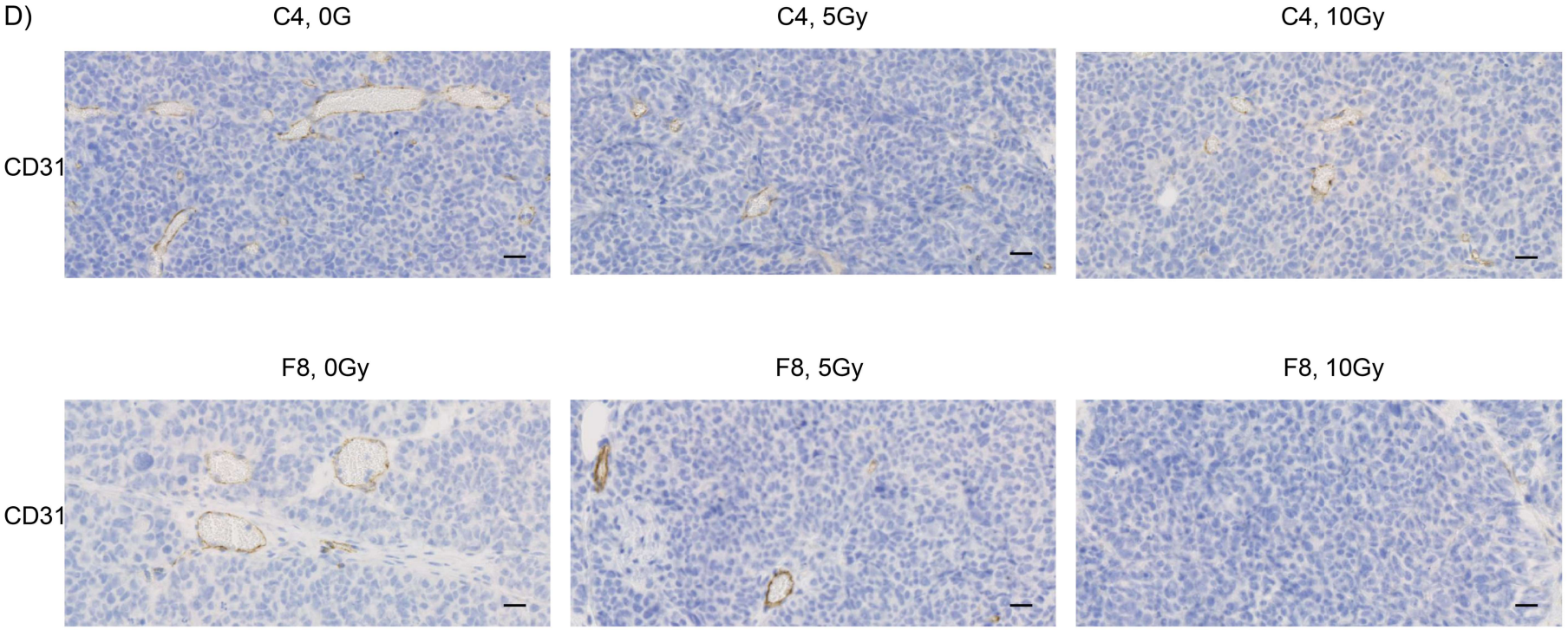
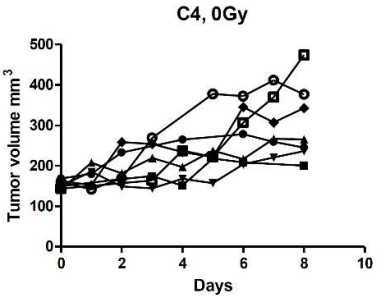
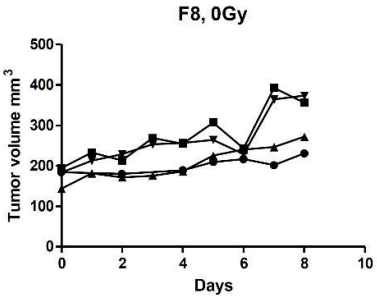
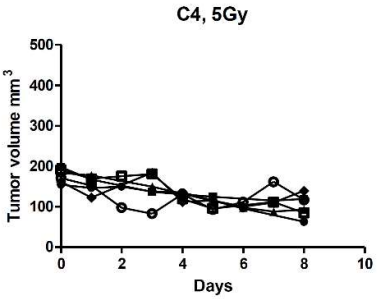
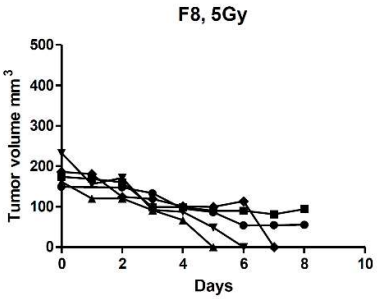


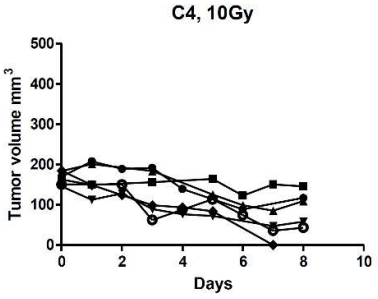
Figure 7

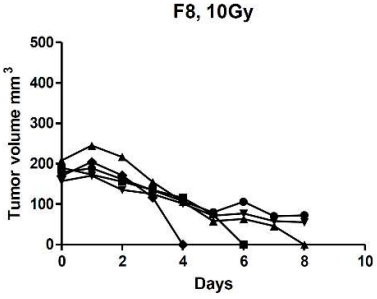

C4, 0Gy
Tumor volume mm3
Days


F8, 0Gy
Tumor volume mm3
Days

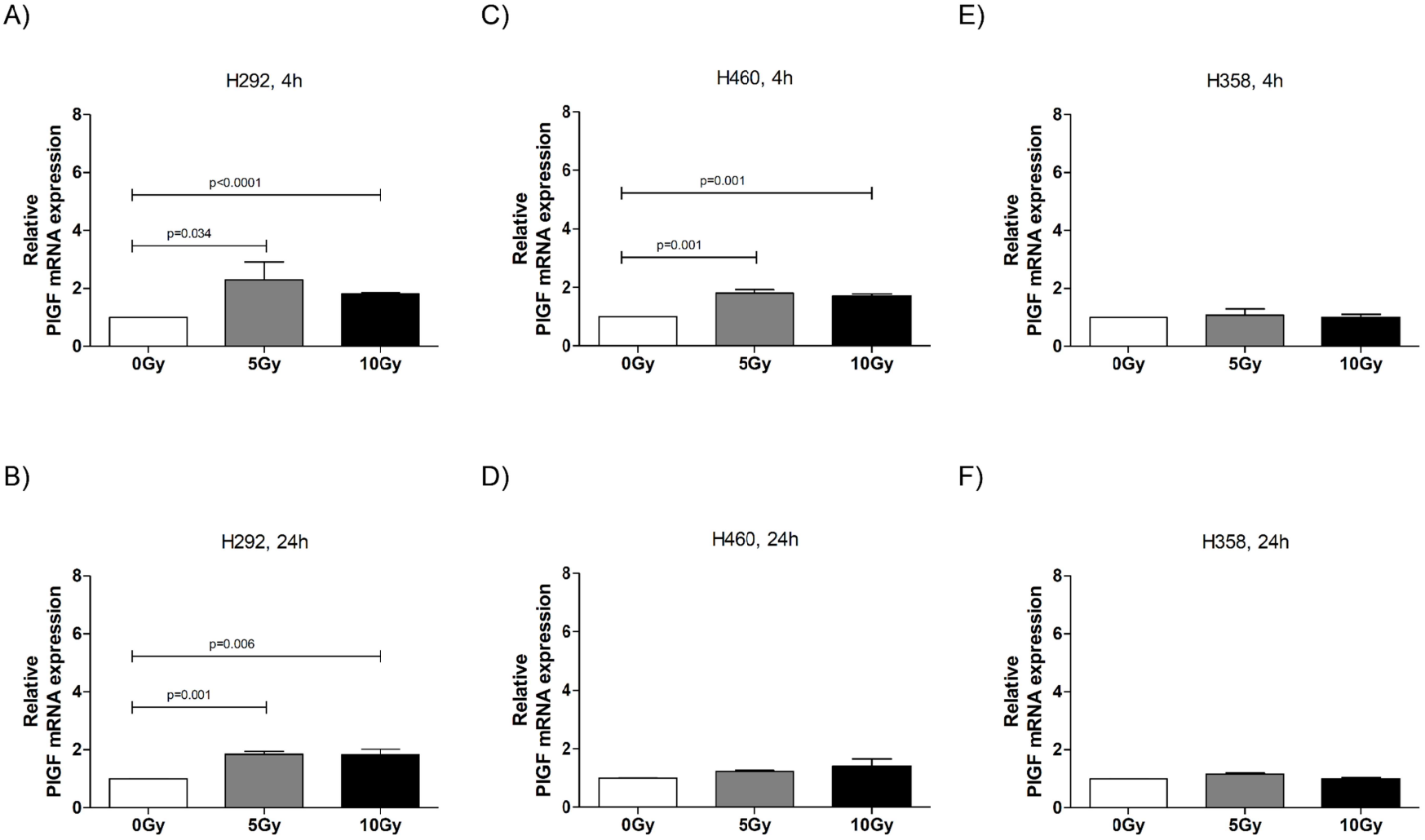

C4, 5Gy
Tumor volume mm3
Days


F8, 5Gy
Tumor volume mm3
Days

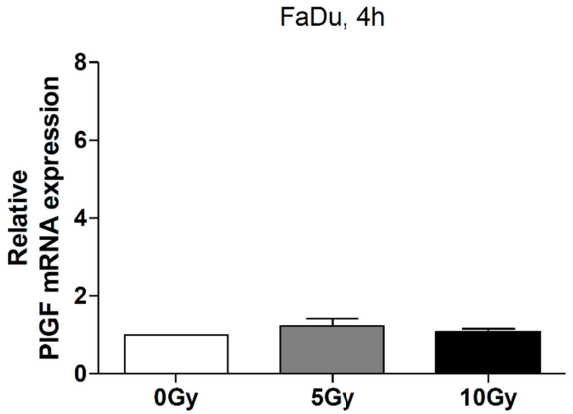

C4, 10Gy
Tumor volume mm3
Days


F8, 10Gy
Tumor volume mm3
Days

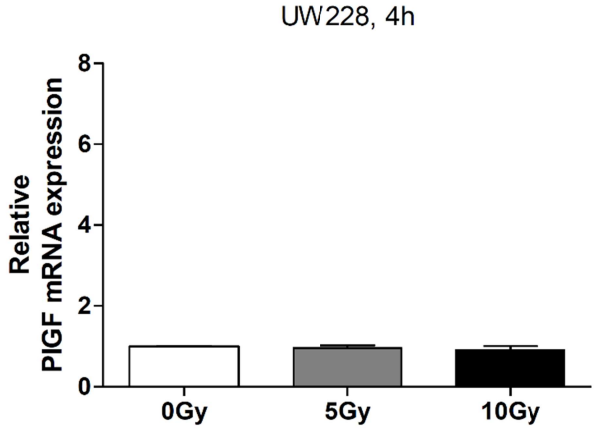
Supplementary Figure 1



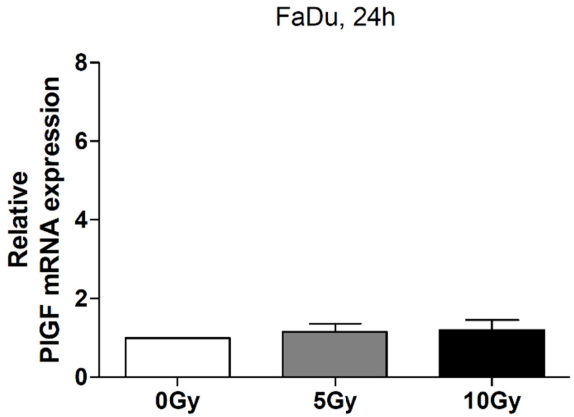
G)


FaDu, 4h
Relative PIGF mRNA expression
0
2
4
6
8
0Gy
5Gy
10Gy

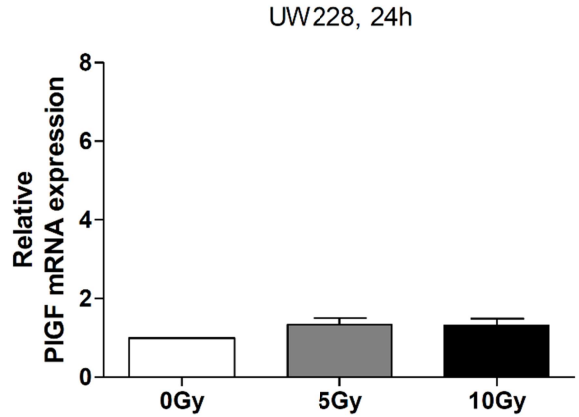
I)


UW228, 4h
Relative PIGF mRNA expression
0
2
4
6
8
0Gy
5Gy
10Gy

H)


FaDu, 24h
Relative PIGF mRNA expression
0
2
4
6
8
0Gy
5Gy
10Gy

J)

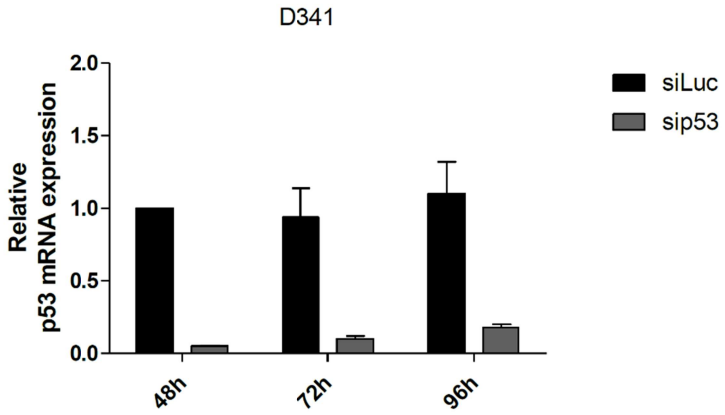

UW228, 24h
Relative PIGF mRNA expression
0
2
4
6
8
0Gy
5Gy
10Gy

Supplementary Table 1

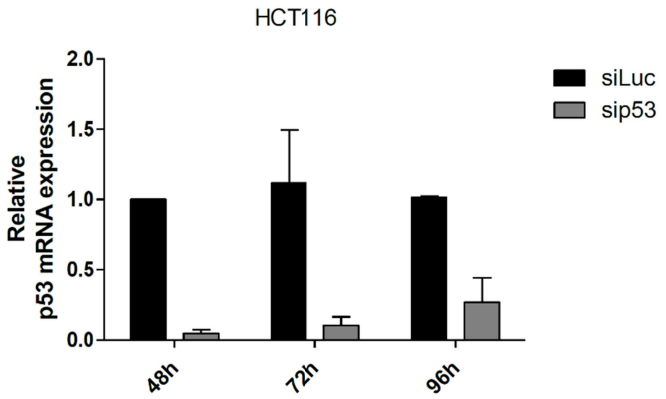
CELL LINES	SOURCE/DISEASE	KRAS STATUS	P53 STATUS	PLGF EXPRESSION AT 4H	REFERENCES
H460	Lung /carcinoma	Q61H	Wild-type	Increased	den Dunnen JT 2000, Leroy B 2017
H292	Lung/carcinoma	Wild-type	Wild-type	Increased	Lu W 2017, Acunzo M 2017
H358	Lung/carcinoma	G12C	Null	Unchanged	den Dunnen JT 2000, Tung MC 2015
FADU	Head and neck/ squamous cell carcinoma	Wild-type	R248L	Unchanged	Somers KD 1992, Hoa M 2002
DAOY	Cerebellum/Medulloblastoma	Wild-type	C242F	Unchanged	Saylors RL 1991
D341	Cerebellum/Medulloblastoma	Wild-type	Wild-type	Increased	Saylors RL 1991
A549	Lung/carcinoma	G12S	Wild-type	Increased	den Dunnen JT 2000, Leroy B 2017
H125	Lung/carcinoma	Wild-type	Frameshift	Unchanged	Phelps RM 1996, Padanad MS 2016
UW228	Cerebellum/Medulloblastoma	Mutated	T155N	Unchanged	Lacroix 2014
HCT116	Colon carcinoma	G13D	Wild-type	Increased	Liu Y 2005, Alves S 2015

Supplementary figure 2

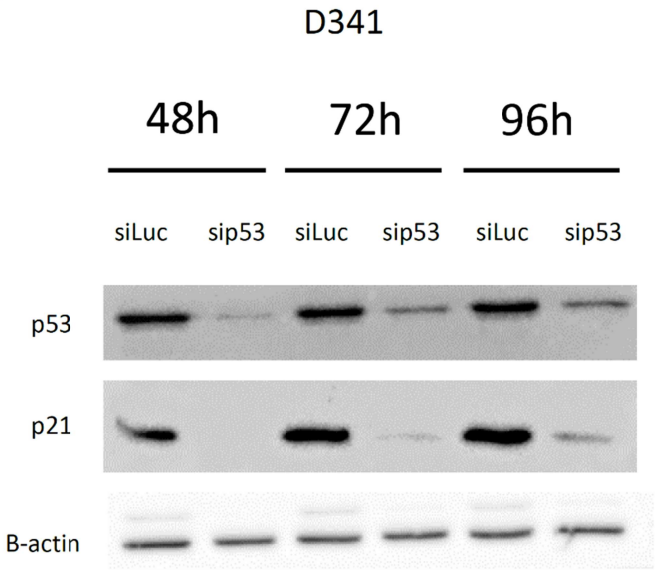
A)



B)



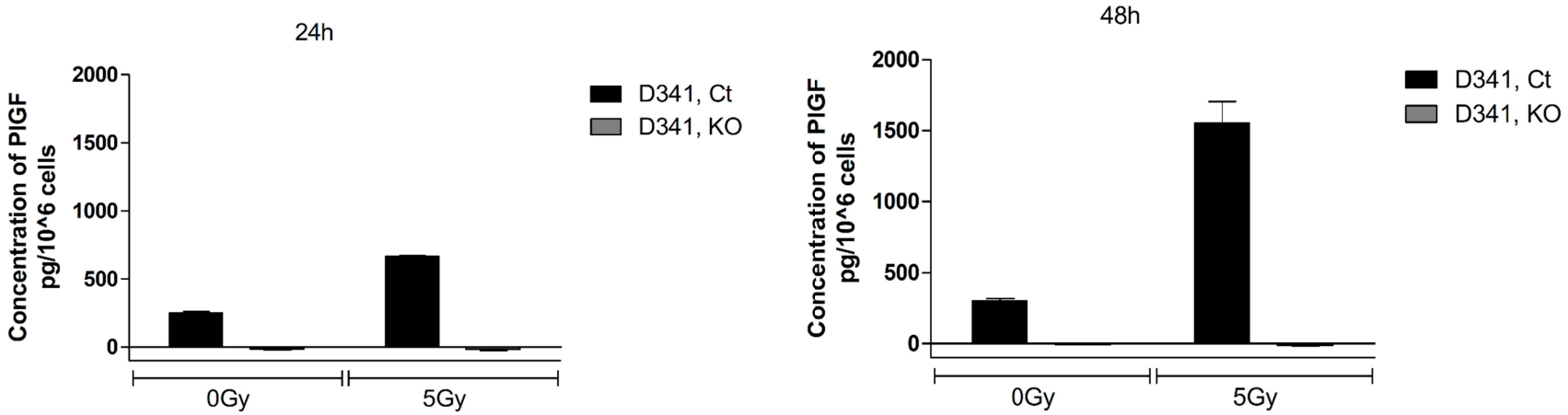
C)



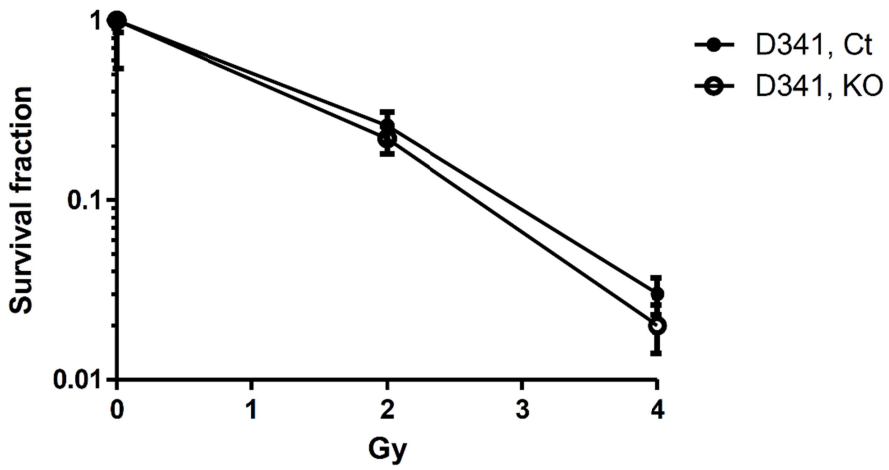
Supplementary figure 3

A)

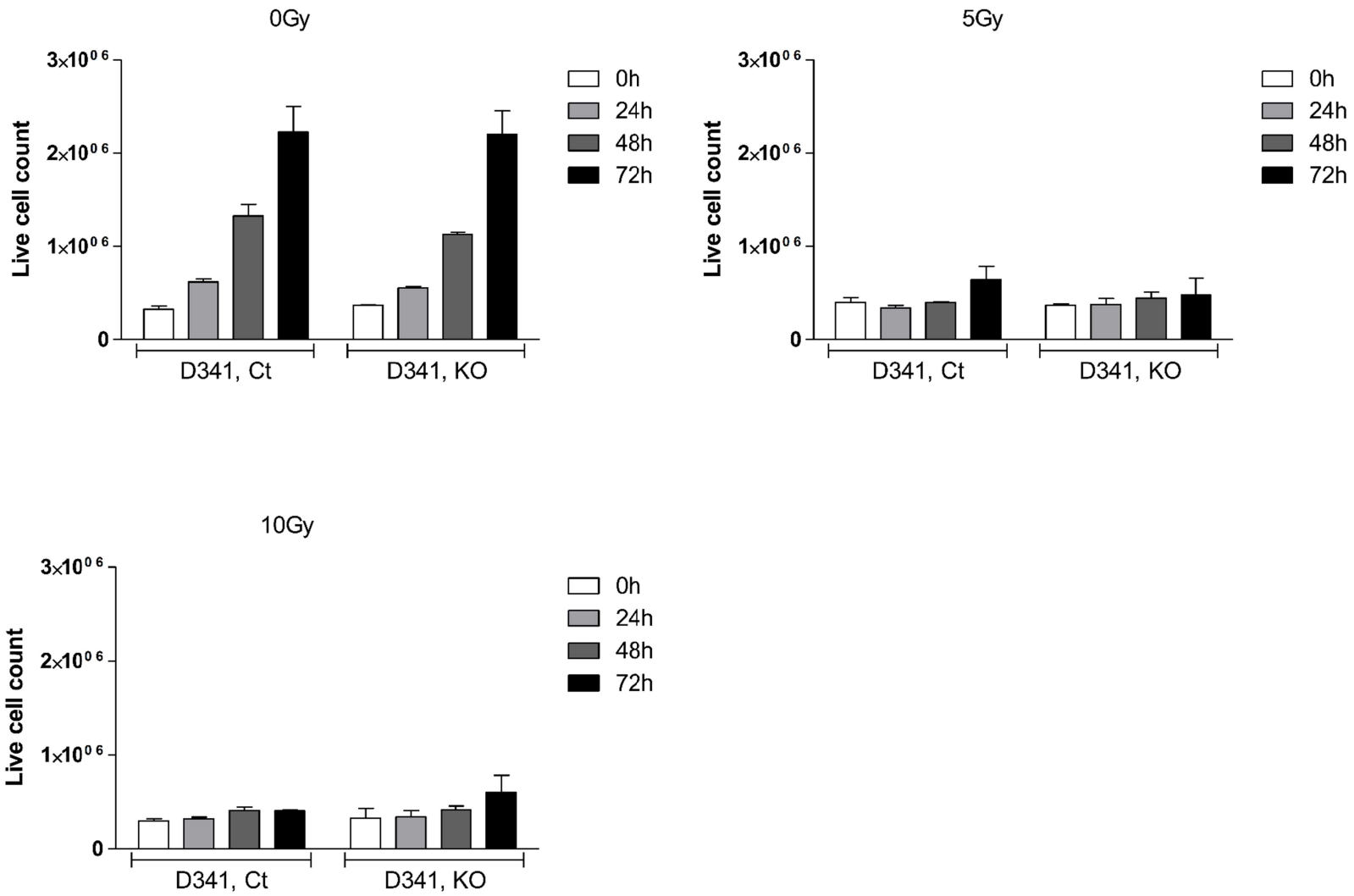
B)


24h
Concentration of PIGF pg/10^6 cells
2000
1500
1000
500
0
0Gy
5Gy
D341, Ct
D341, KO
48h
Concentration of PIGF pg/10^6 cells
2000
1500
1000
500
0
0Gy
5Gy
D341, Ct
D341, KO

C)


Survival fraction
1
0.1
0.01
0
1
2
3
4
Gy
D341, Ct
D341, KO

D)


0Gy
5Gy
10Gy
Live cell count
3×10^06
2×10^06
1×10^06
0
0h
24h
48h
72h
D341, Ct
D341, KO

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4. Discussion

4.1 Challenges in lung cancer and medulloblastoma treatments

Lung cancer is an invasive, fast metastasizing cancer and the main cause of cancer related deaths in both men and women. Incidence and mortality of lung cancer is primarily associated with long term smoking and the susceptibility to carcinogenic substances in tobacco. It is a heterogeneous disease arising at different sites of the lung leading to different symptoms. The majority of patients (70%) are diagnosed at a later stage of the disease, with metastasis found in brain, bone or liver [226]. Lung cancers are categorized into two groups: 1) SCLCs that are centrally located and are the most dedifferentiated. They account for 15% of lung cancers, are aggressive and metastatic. 2) The majority of lung cancers is classified as NSCLC accounting for 85%, with 5-year survival of 16%.

A normal lung cell transforms into a malignant and invasive lung cancer though multiple stages of genetic alterations. The process continually acquiring abnormalities in the genome influences metastasis and treatment resistance [227]. Therefore, it is of great importance and clinical impact to identify and characterize these changes in order to provide better treatment options for patients. Currently, proto-oncogenes such as KRAS, EGFR and HER2 with activating mutations, rearrangements in ALK, several amplifications and gene overexpression among other alterations can be targeted [228]. Lung cancers are usually diagnosed based on symptoms which appear at later stages of the disease and therefore cannot anymore treated with a curative intent. Operable, early-stage NSCLC present 5-year survival of 50-70%. However, these numbers drastically drop in case of advanced disease with metastases [227]. Many attempts have been made in order to identify biomarkers for an early detection of lung cancer. However, currently there are few biomarkers in clinical use for lung cancer detection. Many pharmaceutical agents, such as bevacizumab and TKIs, are approved for lung cancer treatment in combination with radiotherapy. As in many therapeutic approaches for cancer, these treatment regimens lead to resistance and side effects. Thus, there is a need to develop a more personalized and effective therapy that can offer an ideal treatment.

Additionally, tumors can alter the microenvironment to their advantage helping cancer cells evade the immune system. Tumor-associated immune cells help in supporting tumor growth. However, composition of the tumor microenvironment and

the subset of immune cells differ between patients and can decide treatment outcome [229]. Early-stage NSCLCs are primarily treated by surgically removing the tumor and provides best option [230]. In case of unresectable tumors, patients receive radiotherapy, such as SBRT [231]. Treatment modalities for patients with late stage and metastatic disease are radiotherapy combined with chemotherapy, targeted therapy and if possible, surgery [226].

Angiogenesis plays a vital role in many cancers and represents a valuable target, including in NSCLC. One of the main players of angiogenesis, namely VEGF, is approved to be targeted by a monoclonal antibody bevacizumab as a part of first-line treatment in combination with chemotherapy and shows improved response rates in NSCLC patients. However, patients show resistance to this therapy that could be caused by activation of other angiogenic signaling pathways. Several drugs targeting these pathways are currently investigated [232].

Medulloblastoma arises in the cerebellum, at the base of the skull and is the most common childhood brain tumor affecting children between ages of 3 and 9. It accounts for up to 20% of pediatric brain tumors and is a leading cause of cancer-related deaths. Depending on the subtype of the disease, the survival of patients ranges between 40-90% [233]. Medulloblastoma is a highly metastatic disease with symptoms that could lead to misdiagnoses. During medulloblastoma formation, the stem cells in the cerebellum stop diving and differentiating and depending on the molecular pathways affected, the disease can be divided into subgroups [234]. Currently, medulloblastoma is treated by surgically removing the tumor followed by radiation and chemotherapy to reduce metastasis. As mentioned above, children younger than 3 years old do not receive irradiation due to damages to brain. However, this could also aggravate the disease and survival. Hence, there is an urgent need to find new treatments that are well tolerated and cause fewer side effects. Targeted therapies, nanotherapies and inhibitors of involved pathways are developing for more effective way of treatment. An important issue to keep in mind is the ability of these drugs to pass the blood-brain barrier (BBB). BBB has been one of the main reasons to failed treatment for medulloblastoma since many drugs are too large to pass through [235].

Due to different genes and pathways activated, each subgroup has differential response to chemotherapy. Wnt subtype is the most responsive to chemotherapy because of porous vessels with good overall survival rate [235].

Another hurdle to overcome is medulloblastoma stem cells that play an important role in metastasis and resistance, thereby decreasing the survival of patients [236]. In order to develop new drugs, we need to understand the molecular regulation of brain development and the pathways associated with tumor formation.

Drugs used for medulloblastoma treatment can be divided into the following groups: 1) topoisomerase inhibitors (e.g. irinotecan) resulting in inhibition of DNA replication and cell death showing prolonged survival [235], 2) Hedgehog inhibitors such as vismodagib that can bind to SMO and inhibit the activation, subsequently leading to decreased tumor size [237], 3) TKIs (e.g. imatinib) are broadly used to treat many cancer types where they inhibit specific signaling pathways involved in metastasis or cell proliferation [235], 4) PI3K/AKT signaling inhibitors with anti-proliferative effects in combination therapies have shown promising results [238], 5) anti-angiogenic drugs are important in Wnt and Shh subtype as VEGF is their downstream target [239], [235], 6) other small molecules targeting ion channels involved in tumor growth [235].

4.2 The role of the tumor microenvironment in treatment response

The role of the tumor microenvironment (TME) in treatment resistance to radiotherapy has long been investigated. 50% of cancer patients are treated with RT, which not only leads to cancer cell death, but also to stimulation of several processes in the TME. These processes include changes in tumor hypoxia, vascularization and secretion of growth factors to name a few. Each of these processes have been investigated as a potential target for treatment of cancer patients in combination with RT and will be discussed in this section.

Solid tumors contain both normoxic and hypoxic regions. Hypoxia is heavily involved in regulating tumor growth [240] and resistance to RT [241] in these tumors. In response to IR, normoxic tumor cells are killed due to ROS generation and DNA damage. However, hypoxic regions are radioresistant due to lack of oxygen and need almost three times more dose of ionizing radiation as normoxic regions. In order to overcome this hurdle, RT is applied in fractions, killing normoxic cells e.g. around a tumor vessel of the tumor, allowing time for the co-irradiated normal tissue to recover and the hypoxic regions of the tumor to become reoxygenated [242]. One of the main transcription factors upregulated under hypoxia is HIF-1α, which is further increased

after RT and is correlated to poor prognosis in many cancer types, such as head and neck cancer [243]. Many downstream targets of HIF-1α have been identified, including VEGF, which is involved tumor angiogenesis. Another contributor for increased hypoxia in tumors is abnormal tumor vasculature. When oxygen and nutrients do not reach all areas of the tumor due to insufficient blood vessels, HIFs are upregulated in the hypoxic tumor cells, which leads to increased expression and secretion of VEGF in order to stimulate new vessel formation. Therefore, HIFs have become important targets to radiosensitize tumors. Many pharmaceutical agents inhibit its transcriptional activity and subsequently the expression of downstream targets, such as VEGF, further leading to vessel normalization. This in turn leads to increased radiosensitivity and reduced tumor growth [108]. Other hypoxic radiosensitizers, such as Nimorazole, has shown promising results in NSCLC and head and neck cancer patients in combination with chemoradiotherapy [244].

One of the main factors determining the sensitivity to radiotherapy is the availability of oxygen, which is provided by blood vessels. Hyperfractionation with low dose per fraction and a high dose irradiation could have differential effects on tumor vasculature. In response to low-dose hyperfractionated RT, hypoxic tumor cells become oxygenated and tumors regress. However, tumor vasculature is protected in hyperfractionated RT. Strong vascular damage was detected in xenograft models (such as melanoma, glioblastoma) after high-dose hypofractionated RT, which subsequently killed tumor cells. Some studies also show the superiority of hypofractionation to hyperfractionation, suggesting more vascular damage and therefore tumor cell death caused by high dose irradiation. At the same time, tumors irradiated with fractionated RT demonstrated recovered tumor vasculature. Compared to the tumor vasculature, the vasculature in the normal tissue is more resistant to RT, partly due to structure, pericyte coverage and functionality [245]. These studies could in turn explain our current results obtained in PlGF ko xenografts. The unprotected vasculature in PlGF-ko xenografts were more sensitive to high doses of IR and collapsed, leading to regressed tumors.

Tumor cell death by high dose IR also leads to release of tumor antigens, thereby stimulating an immune response [246]. IR on its own can also lead to stimulation of the anti-tumor immune system by upregulating surface markers and release of inflammatory cytokines. On the other hand, low dose hyperfractionated RT rather suppresses the immune system [245]. The effect of single high dose irradiation in

tumor models have extensively shown increased antigen presentation and anti-tumor immune responses, compared to fractionated regimens [245]. Improved CD8+ T cells generation and reduced dissemination due to better antigen presenting ability by dendritic cells was also reported [247]. In addition, the release of pro-inflammatory cytokines in response to high-dose RT augments an anti-tumor immune response [245]. Recent focus has been on targeting inhibitory surface molecules in order to boost the anti-tumor immune system and has been discussed extensively in [248-250]. Even though this combined therapy has shown some success, this thesis focuses on another aspect of combined treatment modality, namely the combination of RT with anti-angiogenic drugs.

Antibodies and TKIs targeting angiogenesis have mainly been directed towards the VEGF/VEGFR pathway in combination with RT and chemotherapy showing reduced hypoxia and vessel normalization in many cancer types [251]. However, there are major drawbacks with these drugs, including normal tissue toxicity and intrinsic or adaptive resistance to therapy. Therefore, scheduling and dose administration of RT and anti-angiogenic drugs is crucial [243]. One of the first anti-angiogenic inhibitors is the anti-VEGF monoclonal antibody Bevacizumab approved for treatment of many cancer types in combination with other drugs.
Tumor vessels usually regress in response to different types of therapies. Therefore, in order to keep growing and maintaining functional vessels, the tumor vasculature depends on pericyte coverage. In tumors treated with drugs against the VEGF/VEGFR pathway, most of the vessels regress, but some remain functional with pericyte coverage. Pericytes in turn secrete enough VEGF to support EC survival. Therefore, targeting pericytes with PDGFR inhibitors in combination with other anti-angiogenic drugs might prove helpful. However, this could in turn lead to metastatic disease due to a disordered vasculature [179].

In our laboratory, we combined Bevacizumab with a novel tubulin binding agent, BAL 27862 and BAL101553, for *in vitro* and *in vivo* studies, respectively. Under hypoxic condition, we observed decreased HIF-1α protein levels, decreased VEGF expression and secretion and inhibited tube formation in three different cell lines *in vitro* after treatment with BAL27862. Our *in vivo* experiments using Bevacizumab in combination with weekly or daily dosing of BAL101553 demonstrated a strong combined effect on tumor growth and vascularization. Furthermore, we combined

Bevacizumab and BAL101553 in paclitaxel and epothilone resistant xenografts and observed a superior anti-tumor effect in combination therapy compared to treatment with a single agent alone. These tumors presented increased tumor necrosis, inhibited proliferation and microvessel density in combination treatments. BAL101553 seems to be a promising agent for a combined treatment with Bevacizumab.

Tumors do not only secrete VEGF, but also other growth factors such as PlGF (will be discussed below) in parallel [252]. Therefore, targeting VEGF might not be helpful, since the other growth factors can stimulate angiogenesis independent of VEGF. Furthermore, patients treated with chemotherapy do often not respond to anti-VEGF therapy. This could imply that tumors that have already received an anti-cancer therapy might have already adapted and thereby are resistant to an anti-VEGF therapy. However, the role of anti-VEGF therapies cannot be neglected in terms of vessel normalization, better oxygen delivery and radiosensitivity in certain cancer types. It also suggests that we should consider other pro-angiogenic factors as possible targets in cancer patients.

4.3 Characterization of PlGF in ionizing radiation regulated secretome

As mentioned above, RT leads to secretion of various growth factors and cytokines as a stress response from tumors. VEGF-A has been the main focus of research in the field of angiogenesis. However, recent investigations show the important role of other factors in angiogenesis, especially in tumors treated with anti-VEGF antibodies.
Two former PhD students from our laboratory performed a semiquantitative large-scale secretome analysis, investigating more than 300 biologically active factors released from A549 lung adenocarcinoma cell line in response to IR. This antibody array-based screen was reevaluated for some factors by a quantitatively more exact bioplex assay. Interestingly, the second member of the VEGF-family, PlGF was strongly increased in response to IR. Compared to VEGF, much less is known about the regulation of PlGF, in particular in response to treatment. Thus, in order to characterize PlGF in tumor cells, we investigated its secretion and expression across multiple cells lines with differential genetic background. Firstly, we observed an early rise of PlGF secretion from some of the investigated cell lines, correlated with increased PlGF expression as early as 4 hours after IR. However, other cells lines investigated only showed minimal or delayed PlGF expression. We hypothesized that

this could depend on the genetic background of the cells and could correlate early PlGF mRNA upregulation with an intact tumor suppressor gene p53. Delayed PlGF expression in the remaining cell lines could be regulated by other transcription factors (TFs), such as HIFs, in p53 mutated cells lines.

Research about the transcriptional regulation of PlGF has been either few or contradictory regarding the regulation by HIF1-α [253, 254] or other transcription factors [255, 256], showing the necessity of further research. The transcriptional regulation of PlGF is not fully understood and several other factors could play a role in regulating its expression. In addition to HIF-1α implications in PlGF upregulation [257, 258], there have been studies reporting binding sites of other TFs, including p53, on the promoter/enhancer region of the PlGF gene. It has four NF-kB and five metal transcription factor 1 (MTF1) binding sites. Regulation of PlGF expression by NF-κB under hypoxic conditions have been demonstrated in HEK cells and could have a pro-angiogenic role. Furthermore, NF-κB is also known to regulate VEGF-A [255]. MFT-1 has been studied in RAS-transformed mEFs under hypoxia, also demonstrated increased PlGF expression [259].

Additional factors with binding sites on the PlGF promoter/enhancer region include Sp1 [257], BF-2 [260] and a predicted HRE. However, cell type and conditions differ between these investigations. Therefore, TFs that can regulate PlGF under hypoxia could be cell type specific. The sole study proposing IR-induced PlGF regulation by p53 is a large-scale transcriptome analysis in normal human B-lymphoblastoid cells. Binding site for p53 on PlGF promoter was confirmed by ChIP assay but was not further investigated [256].

The role of PlGF in tumor growth and metastasis is not fully investigated. Several studies have reported increased PlGF expression in tumor tissue compared to normal surrounding tissue. However, PlGF is not only secreted from tumor cells, but also from macrophages, endothelial cells and other cell types. Furthermore, its expression differs between different tumor types. Based on our *in vitro* secretion data, PlGF seems to be more relevant for treatment response. Therefore, in this PhD-project, we focused on the role of IR-induced PlGF.

4.4 Differential regulation of PlGF and VEGF by TFs

In order to verify that p53 is indeed the main regulator of PlGF in p53 wildtype cancer cells, we used different strategies to target p53 and mechanistically investigated downstream effects on PlGF. First, we aimed to demonstrate that p53 is regulating PlGF independent of IR and therefore chose to activate p53 by other means. p53 is constantly expressed and degraded through ubiquitination in normal and unstressed cells by E3 ligase mouse double minute 2 homolog (MDM2). It promotes degradation of p53 by attaching polyubiquitin chain on lysine residues in the COOH-terminus. This in turn targets p53 for proteosomal degradation. MDM2 can also negatively regulate p53 by directly binding to the NH_2–terminus thereby blocking its transcriptional activity [261]. We used the compound MI-773 for MDM2 inhibition, where MI-773 binds to the same pocket as p53 on MDM2. Following the inhibition, p53 can become activated and regulate the expression of subsequent downstream genes. We observed increase in PlGF expression at 4 hours and secretion at 24 hours similar to IR. Second, we treated cells with the p53 inhibitor PFT-α, which inhibits to p53 by preventing its nuclear translocation and transcriptional activation of downstream genes [262, 263]. Our results demonstrated a decrease in irradiation-induced PlGF secretion in PFT-α pre-treated cells. Many have investigated the mechanism of how PFT inhibits p53, which is still not fully understood. Some experiments show inhibition of DNA binding activity by PFT without any effect on the protein levels of p53, whereas others speculate about PFT targeting a common factor for several pathways or even inhibition after nuclear translocation of p53 [263-265]. Therefore, our next experiment focused on downregulating the mRNA and protein levels of p53 by using an siRNA approach to investigate if this type of specific inhibition of p53 supports our previous results. Both mRNA and protein expression were downregulated in sip53 treated cells, also showing p53-dependent downregulation of p21. We observed significant decrease in IR-induced PlGF secretion in p53 downregulated cells.

Our next question rose from our previous experiments with MI-773. We speculated that as an inhibitor of MDM2, MI-773, could also lead to activation of other factors (such as MYCN, Smad3/4, NF-κB) that are usually inhibited by MDM2. The PlGF promoter has additional binding sites for other transcription factors than p53, such as NF-kB as mentioned above, that could also potentially be affected by MDM2. To demonstrate that p53 is the main TF relevant for IR-mediated PlGF regulation, we

treated p53 downregulated cells with MI-773. PlGF expression and secretion remained downregulated in sip53 treated cells even in the presence of MI-773.

Our demonstration of early, IR-induced and p53-dependent upregulation of PlGF-expression was absent in p53-mutated cells. Nevertheless, PlGF-expression was also present in p53-mutated tumor cells in response to increasing doses of IR but only at later time points. We therefore hypothesized that this effect might depend on other signaling pathways and aimed to investigate different signal transduction pathways that could potentially play a role. We decided to investigate hypoxia dependence by using the pharmaceutical agent BAY 87-2243. BAY 87-2243 is a mitochondrial complex I inhibitor and blocks the production of reactive oxygen species, which leads to HIF1-α inhibition [266]. Although there have been contradicting results regarding the link between HIF-1α and PlGF [253, 254, 257, 267-269], we observed significant downregulation of PlGF secretion with the HIF-1α inhibitor BAY 87-2243 under hypoxia in p53-mutated DAOY cells. p53 wildtype D341 cells showed an IR-dependent increase in PlGF secretion. However, this could not be inhibited by BAY 87-2243 under normoxia or hypoxia. We downregulated p53 in D341 cells to examine whether PlGF secretion could further be inhibited after BAY 87-2243 treatment and thereby show hypoxia dependence after p53 inhibition. We did not observe additional decrease in PlGF secretion (data not shown). This could be due to insufficient p53 downregulation and/or the competition between p53 and HIF-1α. Based on these results, p53 seems to be the dominant regulator and further support our previous experiments on p53 dependency in D341 cells. Additionally, these results could also explain the inconclusive reports about PlGF regulation by HIF-1α.

By activating and secreting angiogenic factors, tumor cells can adapt to hypoxic conditions as the tumor grows [270]. Expression and secretion of one of the main angiogenic factors, VEGF, is low under normoxia and increases in response to hypoxia. Decreased oxygen concentration stabilizes HIF-1α, which in turn binds to the HRE of VEGF leading to its activation and secretion [271]. Tumors become more invasive and metastatic. Therefore, the VEGF signaling pathway has become a major target for anti-angiogenic drugs. However, in response to anti-angiogenic therapies, tumor cells upregulate other angiogenic factors, such as PlGF.

Compared to PlGF regulation, wildtype p53 controls VEGF expression in an opposite way. According to some studies, wildtype p53 suppresses VEGF production by binding

and inhibiting HIF-1α during hypoxia [272, 273], although this has been contradicted by others [274]. In addition, in vivo models have shown increased VEGF production, tumor progression and increased angiogenesis in response to p53 loss of function. This in turn was reported to be due to the inability of mutant p53 to regulate HIF-1α [271]. Loss of p53 also supports NF-kB expression and inflammation, and leads to cancer metastasis. Furthermore, co-culturing of CAFs with p53 knockout HCT116 cells increased VEGF mRNA and protein expression in CAFs. CAFs are important part of the microenvironment and could potentially play a vital role in treatment response. We do not yet fully understand the interaction between CAFs and tumor cells, but it offers an advantageous microenvironment for cancer cells. Consequently, CAF levels are considered as indicators of poor prognosis in many cancer types. Taken together, these reports suggest a tumor promoting microenvironment due to loss of p53 [275]. On the other hand, wildtype p53, inactivates HIF-1a and therefore suppresses VEGF expression leading to decreased vascularization.

According to our own results, in a p53 mutated background, HIFs are the major driving force for PlGF, which is similar to VEGF regulation. Therefore, BAY 87-2243 can also downregulate (IR-induced) expression of PlGF, which still needs to be tested for VEGF. Compared to VEGF, wildtype p53 dominantly drives PlGF expression, which is strongly activated e.g. by external stimuli such as ionizing radiation. PlGF in turn could upregulate VEGF expression in the tumor. Therefore, it is possible, that when patients are treated with an anti-VEGF directed treatment modality, the early response is vessel regression. However, due to anti-angiogenic agent, wildtype p53 status and PlGF secretion, these tumors might become resistant to therapy. The regulation of PlGF and VEGF by the same TFs seems to be very different. These results indicate that we should consider the status of p53 in cancer cells more seriously and adjust treatment forms targeting pro-angiogenic factors.

4.5 PlGF as a promising target for combined treatment modality

PlGF has been suggested as a target for anticancer therapy due to its role in angiogenesis and inflammation [165]. Blockade of PlGF by neutralizing antibodies [164, 171, 181, 182] or gene inactivation [110, 174] resulted in reduced angiogenesis, inflammation, metastasis and tumor growth in several tumor models. Furthermore, genetic or pharmacological targeting of PlGF in medulloblastoma models showed

reduced tumor growth [183]. Even in spontaneous and transgenic tumor models, PlGF targeting demonstrated similar results as the studies mentioned above [174]. Additionally, a combination of anti-PlGF-directed with anti-VEGFR2-directed antibodies resulted in a stronger effect compared to monotherapies and also reduced tumor growth in anti-VEGFR2 resistant tumors [164] suggesting its significance as a promising target in a combined treatment modality. Apart from vessel normalization, inhibition of PlGF also improved the effect of chemotherapeutic agents used in combination therapy [164]. However, there are contradicting studies showing no antitumor effect of PlGF inhibition with neutralizing antibodies [174, 185, 276]. It is important to keep in mind that neutralizing antibodies might not have the same effect in vivo as in vitro. Furthermore, the response to neutralizing antibodies may depend on the expression of VEGFR1.

In the second part of this project, we aimed to study the role of PlGF as a part of combined treatment modality in tumor angiogenesis and radiosensitivity. With complementary in vitro as well as in vivo experiments, we investigated the effect of PlGF targeting in combination with IR on tumor vascularization and radiosensitivity.

We successfully generated GFP-expressing two PlGF wildtype control cell lines (PlGF-wt) and four PlGF kockout cell lines by clustered regularly interspaced palindromic repeats (CRISPR)/Cas9 strategy. The target sequence for the first sgRNA contained a cysteine residue involved in intrachain disulfide bond, which plays an important role for the stability of the protein. PlGF secretion in all six cell lines was determined through ELISA and demonstrated undetectable levels of PlGF in knockout cells lines, even in response to IR (not included in manuscript). To investigate the paracrine role of PlGF, we co-cultured PlGF-wt and PlGF-ko cells with HUVECs and investigated the migratory capacity of HUVECs towards non-irradiated or irradiated cells. We observed increased number of migrating HUVECs towards irradiated PlGF-wt cells in response to IR. Number of migrating HUVECs towards PlGF knockout cells did not change. These results demonstrate a specific PlGF dependent paracrine effect in vitro and indicate that PlGF could be in particular important for radiotherapy-regulated tumor angiogenesis and could thereby also co-determine the treatment efficacy.

Our *in vivo* experiments demonstrated similar MVD in both PlGF-wt and PlGF-ko xengtrafts. In reponse to irradiation, MVD minimally decreased in PlGF-wt xenografts. However, PlGF-ko xenografts revealed strongly reduced MVD in response to high dose irradiation. As an indicator of pericyte coverage and vessel functionality,

we assessed smooth muscle actin (SMA) staining in xenografts. We observed decreased pericyte coverage and increased radiosensitivity in irradiated PlGF-ko xenografts. Additionally, PlGF-ko xenografts treated with 5 or 10Gy showed regressed tumors in 60% of cases for each treatment group. These results in part support previous reports about a partially reduced vascular formation in PlGF-ko mice [174]. However, to our knowledge, targeting of PlGF in combination with IR has not been investigated so far. Based on our results, PlGF plays a protective role and could be a part of a rescue mechanism in response to IR, especially in the early time point of treatment.

These results support the concept that PlGF plays a similar role as VEGF as previously demonstrate. However, one of the main differences between these two angiogenic factors is their expression. VEGF-A is always present in normal tissues and important for normal angiogenesis. It also acts as a chemoattractant for endothelial cells and plays a major role in new vessel formation after exercise. Therefore, targeting VEGF-A leads to normal tissue toxicities and treatment resistance. In contrast, PlGF does not regulate or maintain normal vessel formation in adults and is upregulated only during disease. It is usually associated with poor disease outcome in several cancer types. PlGF only binds to VEGFR1 and with higher affinity, which means VEGF-A is displaced and available for VEGFR2. Thereby, a parallel and stronger angiogenic signaling takes place. This issue could in turn explain why anti-VEGF therapies show promising results at first, but fail over time due to presence of another strong angiogenic signaling, namely by PlGF. Furthermore, targeting VEGF-A further increases PlGF expression and a subsequent angiogenic signaling, where PlGF can act as a chemoattractant for other cells, such as macrophages, endothelial cells and pericytes. Pericytes recruited to newly formed vessels due to increased PlGF secretion leads to vessel protection and maturation. Our own results have shown decreased pericyte coverage and increased radiosensivity in irradiated PlGF-ko xenografts. PlGF also activates differentiation of macrophages into M2-like phenotype, which supports tumor growth by secreting more angiogenic factors [277]. PlGF is also known to increase VEGF-A secretion from mononuclear cells. Therefore, inhibiting PlGF would in turn decrease VEGF induced angiogenesis.

Resistance to different treatment modalities, including radiotherapy, is one of the main drawbacks for cancer patients. There is a need to identify new biological markers that can be targeted in combination with radiotherapy. Patients treated with anti-

angiogenic drugs combined with chemoradiotherapy often present increased PlGF levels, vessel number and permeability, which correlates with poor survival. However, inhibitors of PlGF have demonstrated reduced tumor vascularization without affecting healthy vasculature *in vivo*. All these results suggest PlGF as a promising target in cancer treatment. Though, PlGF neutralizing antibodies did not show improved survival in combination with Bevacizumab in previously treated glioblastoma patients [164, 167, 171]. However, PlGF might be a highly relevant target as part of a combined treatment modality, in which its "combination partner" induces the expression and secretion of PlGF as part of a protective stress response. Targeting of PlGF might thereby reduce an induced treatment threshold and sensitize for the damaging part of the "combination partner", in this regard to radiotherapy. At the same time, important aspects to consider before treating patients with anti-PlGF antibodies alone or more favorable as part of a combined treatment modality, are the determination of the p53 status of patients and previous treatments for determining therapy resistance in order to offer the best combined treatment option.

5. Outlook

Approximately 50% of all cancer patients receive radiotherapy alone or in combination with other treatment regimens. Although ionizing radiation (IR) causes DNA damage and tumor cell killing, it also leads to stress responses from tumor cells leading to secretion of multiple factors and subsequently resistance to therapy. In this PhD thesis, we have shown that one of the factors secreted from cells lines derived from different tumor entities in response to increasing doses of IR is placental growth factor (PlGF). Not only secretion, but also mRNA expression of PlGF is increased across multiple cell lines, where early increase in mRNA expression was related to the transcriptional activity of the tumor suppressor p53.

We have provided insights into regulation of PlGF by wildtype p53 through mechanistic investigations that have not been shown before. Unfortunately, we were unable to detect p53 binding sites on the PlGF promoter. This could be due to technical challenges, due to cell type difference and/or different p53 binding sequences. Therefore, it will be important to perform additional rounds of extended ChIP assays with multiple non-irradiated and irradiated p53 wildtype transformed und untransformed cells to detect specific binding of wildtype p53 to the promoter region of PlGF in order to further support our data.

We briefly investigated PlGF regulation in p53 mutated cells under hypoxia and normoxia. Although our current experiments need to be corroborated with additional control experiments. We could demonstrate increased PlGF secretion under hypoxic conditions in p53 mutated cells in response to irradiation. This was in turn abrogated after treatment with the HIF-inhibitor BAY 87-2243. Future experiments for this part of the project should include investigations of PlGF expression and secretion in additional p53 mutated cell lines in response to hypoxia and IR with or without different classes of HIF inhibitors. As we cannot exclude additional roles of other transcription factors (TFs) in the regulation of PlGF expression, e.g. mutant p53, MTF1 etc., additional experiments with different levels of hypoxia should be considered in order to understand the interplay between HIFs, p53 and other TFs. It is also important to distinguish between HIF-1α, HIF-2α or HIF-3α as the main regulator of PlGF under these conditions, e.g. using different HIF-ko systems or HIF-isoform specific downregulatory si/sh-constructs. Overall, mechanistic experiments dissecting the role of different, in part competing transcription factors under different conditions

and in different cell systems will be next steps to be addressed in order to gain deeper insights into the regulation of PlGF in response to irradiation.

An additional readout for both *in vitro* and *in vivo* experiments is macrophage polarization. Our aim is to collect blood from healthy donor, isolate monocytes from the whole blood and polarize them to the M0 phenotype. Using conditioned media (CM) derived from our irradiated and non-irradiated PlGF knockout cells in parallel with positive and negative controls we can examine whether t M0 macrophage change their phenotype to M2-like macrophages by investigating specific gene expression in these macrophages. For *in vivo* experiments, we propose staining of tumor slices derived from our xenograft model with antibodies directed against surface markers for investigation of macrophage infiltration.

The importance of PlGF for the treatment response to radiotherapy and IR-induced tumor vascularization remains crucial. PlGF is the lesser investigated member of the VEGF-family and to our knowledge, there are no existing data showing its IR-induced role and relevance neither *in vitro* nor *in vivo*. To assess this, we successfully generated PlGF knockout cells using the CRISPR/Cas9 system. PlGF knockout cells did only show minor differences in proliferative activity or clonogenecity compared to control cell lines *in vitro*. Our investigations of the paracrine role of PlGF with the migration assay with human umbilical vein endothelial cells (HUVECs) demonstrated increased migration of HUVECs towards irradiated control cells but not irradiated PlGF knockout cells. Additionally, our *in vivo* experiments revealed a corrupted tumor vasculature and increased tumor regression in PlGF-ko xenografts in response to single high doses of irradiation. increased radiosensitivity. It will be relevant to confirm these results with additional PlGF knockout cell lines, injected either as a single clones or as mixed clone populations. Furthermore, it will be of interest to perform similar *in vivo* experiments with tumor cells derived from other tumor entities and not only in response to single high doses of irradiation but also with fractionated treatment regimens. Eventually the role of PlGF for the treatment response to radiotherapy will have to be investigated in more advanced complex tumor models, e.g. orthotopic tumor models in an immunocompetent background. Like the relevance of IR-induced PlGF for the treatment response will be evaluated with PlGF-directed inhibitory antibodies.

PlGF is involved in pathological angiogenesis and it is of high translational relevance. Many cancer patients show increased levels in response to various treatments. Therefore, it will be of interest to obtain patient samples with known genetic backgrounds (specifically with regard to p53) for detailed investigations. Overall, all these experiments will further explain the regulation of PlGF and its IR-induced role and relevance in a translational setting.

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Acknowledgements

I would like to thank many people for the success of this PhD project:

First and foremost, I would like to thank Prof. Dr. Martin Pruschy for this immensely interesting project and for choosing me as a candidate to work in his laboratory. I grew to love this project every year and it is in part thanks to all the motivation, criticism, mentoring and guidance I received from Martin. In line with this, many thanks go to my committee members Prof. Dr. Beat Schäfer, Prof. Dr. Michael Detmar, PD. Dr. Martin Baumgartner and PD. Dr. Oliver Riesterer for making time for the meetings, for their useful suggestions and for pushing me and this project further. Thank you all for improving this project.

I would like to thank former and current lab members: Ashish Sharma, Sabine Bender, Ivo Grgic, Fabienne Tschanz, Verena Waller, Irma Telarovic, Danielle Villars, Simon Deycmar, Philip Knobel, Erica Faccin and Carmen Yong for their company and support. It has been a great working atmosphere. Thanks to Fabienne for performing the migration assays and proofreading. Special thanks to Ashish for his help and guide in the beginning of my thesis and teaching me some of the methods in the lab. The PlGF project started because of the secretome analysis performed in part by Ashish.

A big thank you to the Institute of Physiology for being so welcoming and enjoyable environment. Our TGIFs and Secret Santa events have made it easy and fun to get to know you.

Also, thanks to our collaborators from Prof. Dr. Beat Schäfer's lab, Dr. Marco Wachtel and Gloria Pedot for their help with CRISPR/Cas9 system and sorting cells, respectively.

Many thanks go to my closest friends from Sweden, Daniela, Dodona, Emma, Sara, Sivan and Ivana for always being interested in my work, for supporting me and for making me feel good every time I talked to you. Thanks for all the visits and the fun you brought with you! I would also like to thank all the friends I met in Zurich for their help in balancing professional and personal life. It has been a pleasure to know you all and I am happy to have shared this experience with you.

This project has been a part of Marie-Curie ITN RADIATE program for three years, where I met so many talented young researchers. The workshops were always fun and

informative with lots of ideas and support for the projects. I am privileged to have been a part of such a group and I thank all of them for the three years we spent together as part of the program.

I would like to thank my boyfriend Simon Gnädinger for making everything so much easier. Without him the last part of my thesis would have been even more stressful. Thank you so much for the support and the laughter!

Last but not least, my deepest thanks go to my family: Stort tack till mina syskon Tahmina och Sahib, för att ni har varit allt en storasyster kunde begära. Ni har gett otroligt mycket glädje till mit liv och utan er hade jag nog inte klarat det här. Ni har varit en stor inspirationskälla för mig och tack så mycket för att ha varit där för mig i svåra tider. Mamma och pappa, tack för allt ni har gjort och uppoffrat för min skull. Tack för att ni har alltid funnits där och stödjat mig i mina val. Tusen tack för att ni har alltid prioriterat era barn och deras utbildning. Utan er hade inget av detta hänt och det är jag evigt tacksam för. Jag kunde drömma större och nå högre på grund av er. Jag älskar er alla!

Curriculum Vitae

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Date of birth: 2. Nov 1989

Nationality: Swedish

EDUCATION

Nov 2015 – January 2020	**PhD student in Cancer Biology, University of Zurich**	**Zurich**
Aug 2012 – June 2014	**Master of Science, Uppsala University**	**Uppsala**
Aug 2009 – June 2012	**Bachelor of Medical Science, Karolinska Institute**	**Stockholm**

EMPLOYMENT HISTORY

June 2011 – Sep 2015	**Biomedical laboratory Scientist, Karolinska Hospital**	**Stockholm**
June 2010 – May 2011	**Barista and Cashier, Cafe Bellman**	**Stockholm**
Oct 2010 – May 2010	**Private tutor, Matteakuten Studybuddy**	**Stockholm**

PROJECTS DURING STUDIES

Jan 2014 – Aug 2014 **University of Manchester, Manchester**
Thesis project performed at Prof. Michael Lisanti's lab at Breakthrough Breast Cancer concentrating on cancer metabolism, microenvironment and Reverse Warburg effect. I studied the role of mitochondrial fusion and fission genes overexpressed in immortalized fibroblasts and how this overexpression can lead to the production of energy metabolites. The effect of this overexpression on mitochondrial dysfunction was also studied. Improved laboratory skills including cell culture, flow cytometry, western blotting and number of different assays (e.g. Lactate, ketone, ATP etc.) were acquired.

Mar 2012 – June 2012 **Karolinska University Hospital, Stockholm**
Thesis project performed at Transfusion Medicine laboratory at Karolinska Hospital in order to find new screening cells specifically for RhD negative pregnant women who had received Rh-prophylaxis. I was able to identified 4 new screening cells from donors allowing to find other clinically important antibodies in mothers. These screening cells are routinely used in three major hospitals.

Sep 2011 – Dec 2011 **University of Texas Health Science Center, San Antonio, Texas**
Exchange studies with clinical courses done at the University Hospital laboratories: hematology (4 weeks), immunology (2 weeks) and microbiology (4 weeks). Also, two weeks of environmental course in Laredo, including air and water safety and family visit.

LANGUAGES

English (Fluent)	Azerbaijani (Native speaker)	Swedish (Fluent)
German (B)1	Turkish (B1)	Russian (B1)

FURTHER ACTIVITIES

RADIATE Marie-Curie Innovative training network (ITN), 2015-2018

3 years of PhD-studies were part of Marie-Curie ITN (http://www.radiate.eu/), a European Research project. The network included seven universities across Europe with two students each. My project has been part of "New therapeutic strategies for radiotherapy" section. There have been six symposia in total, where I have had both oral and poster presentations about the progress of my project. Some of the meetings were combined with other conferences, where I also have presented my work. These meetings have led to networking and collaborations with other partners.

Global health course in Tanzania (through Karolinska Institute), 2012

Global Health course (4 weeks) divided to two weeks of theoretical course in Stockholm and two weeks of practical course in Tanzania, with visits to different hospitals, laboratories and families in Dar es Salaam and Bagamoyo. Some environmental expeditions were also included.

Oral presentations

1st Comprehensive Cancer Center Zurich Scientific Retreat, Emmetten, 2019

Kolloquium in angewandter Krebsforschung, Universitätsspital Zurich, 2018

Marie Curie ITN symposium in Maastricht, 2018

7th Cancer Biology PhD program retreat, Ascona, 2018

Kolloquium in angewandter Krebsforschung, Universitätsspital Zurich, 2017

7th Cancer Network Zurich retreat, Emmetten, 2017

Marie Curie ITN symposium in Oxford, 2016

Poster presentations

ESTRO meeting in Milan, 2019

International Marie Sklodowska-Curie Meeting in Paris, 2018

ERRS/GBS conference in Essen, 2017

Marie Curie ITN symposium in Essen, 2017

Marie Curie ITN symposium in Zurich, 2016

6th Cancer Biology PhD program retreat, Davos, 2016

AWARDS

Grant from Hartmann Müller – Stiftung